# Investigating the role of Epstein-Barr Virus in the pathogenesis of Multiple Sclerosis.

**Kaija Strautins (BSc. Hons)**

**PhD Candidate**

**This thesis is presented for the degree of Doctor of Philosophy of Murdoch University**

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# Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Kaija Strautins

21st August, 2015

# Acknowledgements

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# Dedication

This thesis is dedicated to my parents. I couldn't have finished this without your emotional, financial and psychological support. Dad, I like to think I got my passion for science and constant desire to learn from you. Mum, you are an extraordinary role model for academic achievement, and you always got me to keep my eye on the end result with balanced optimism and practicality. I love you both. Thank you.

# Abstract

Multiple Sclerosis (MS), a neuronal demyelination disease of the central nervous system, is the most common neurological disease in young adults worldwide, with an unknown cause. It affects over 23,000 people in Australia, and despite effective immune-based treatments, clinical deterioration and disability still occur. Epstein-Barr Virus (EBV), a human herpesvirus, which infects early in life and remains dormant within immune cells, is common in the unaffected population but almost universal in MS patients. Studies have found that MS patients maintain elevated EBV-specific antibody levels, most notably against latent Epstein-Barr Nuclear Antigen-1 (EBNA-1) proteins. The aims of this research were to investigate the significance of EBV immune responses, including those targeting the novel epitope EBNA-$1_{(398\text{-}413)}$, previously associated with MS risk in disease-discordant identical twins, and to understand the relationship between EBV-specific serological responses and genetic risk factors in an established MS cohort (n=426) and healthy controls (n=186). This study also investigated the influence of arginine modification (citrullination) on EBNA-$1_{(398\text{-}413)}$-specific antibody responses, as well as the role of EBNA-1-specific IgG subclass bias. Novel in-house assays were compared with commercial ELISAs for EBV viral capsid antigen (VCA) and EBNA-1. MS patients had significantly higher antibodies against all EBV targets. Inclusion of EBNA-$1_{(398\text{-}413)}$-specific antibody levels further discriminated cases and controls in risk analysis, in addition to genetic risk factors. Citrullinated and IgG1-specific EBNA-1 antibody levels were also elevated in MS cases compared to controls, although they did not improve case-control classification in combined statistical models. EBNA-$1_{(398\text{-}413)}$-specific IgG from acute patients showed different protein reactivity from whole serum, as well as differences between progressive MS serum and healthy control plasma. This project contributes to the importance of EBV in the pathogenesis of the complex disease MS, and has identified novel and statistically powerful relationships between environmental and genetic MS risk factors.

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# List of Units

| | |
|---|---|
| % | Percentage |
| cm | Centimetre/s |
| $^oC$ | Degree/s Celsius |
| h | Hour/s |
| µL | Microlitre/s |
| mL | Millilitre/s |
| µg/ mL | Microgram/s per millilitre |
| mg/ mL | Milligram/s per millilitre |
| mM | Millimolar |
| M | Molar |
| n | Sample size |
| U/ mL | Units per millilitre |
| *g* | Centrifugal acceleration |
| rpm | Revolutions per minute |
| MD | Mean Difference |
| *log* | Logarithm (base 10) |
| p | Statistical significance level |

# Abbreviations

| | |
|---|---|
| aa | Amino acids |
| αßC | Alpha Beta Crystallin |
| ADCC | Antibody-Dependent Cell mediated Cytotoxicity |
| ANOVA | Analysis of Variance |
| APP | Antigen Processing Pathway |
| BAFF | B cell Activation Factor |
| BBB | Blood-Brain Barrier |
| BCR | B Cell Receptor |
| BMP | Bitmap Image |
| BSA | Bovine Serum Albumin |
| CD | Cluster of Differentiation |
| CIS | Clinically Isolated Syndrome |
| CSF | Cerebrospinal Fluid |
| CNS | Central Nervous System |
| $CO_2$ | Carbon Dioxide |
| CTL | Cytotoxic T Lymphocytes |
| DIS | Dissemination in Space |
| DIT | Dissemination in Time |
| DMEM | Dulbecco's Modified Eagle Medium |

| | |
|---|---|
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribose Nucleic Acid |
| EAE | Experimental Autoimmune Encephalomyelitis |
| EBER | Epstein-Barr virus Encoded small RNA |
| EBV | Epstein-Barr Virus |
| EBNA | Epstein-Barr Virus Nuclear Antigen |
| ECL | Enhanced Chemiluminescence |
| EDSS | Expanded Disability Status Scale |
| EDTA | Ethylenediaminetetraacetic Acid |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FBS | Foetal Bovine Serum |
| FDA | Food and Drug Administration (United States of America) |
| FLAIR | Fluid Attenuated Inversion Recovery |
| GAr | Glycine-Alanine Repeat |
| Gd | Gadolinium |
| GFAP | Glial Fibrillary Acidic Protein |
| GWAS | Genome Wide Association Study |
| HHV | Human Herpes Virus |
| HLA | Human Leukocyte Antigen |
| HSV | Herpes Simplex Virus |

| | |
|---|---|
| IC | Internal Control |
| IFNγ | Interferon gamma |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin Gamma |
| IIID | Institute for Immunology and Infectious Diseases |
| IL | Interleukin |
| IM | Infectious Mononucleosis |
| LCL | Lymphoblastoid Cell Lines |
| LMP | Latent Membrane Protein |
| MBP | Myelin Basic Protein |
| MHC | Major Histocompatibility Complex |
| MOG | Myelin Oligodendritic Glycoprotein |
| MRI | Magnetic Resonance Imaging |
| mRNA | Messenger Ribose Nucleic Acid |
| MS | Multiple Sclerosis |
| MSIF | Multiple Sclerosis International Federation |
| MSSS | Multiple Sclerosis Severity Score |
| NK | Natural Killer |
| OCB | Oligoclonal Bands |
| OD | Optical Density |

| | |
|---|---|
| OND | Other Neurological Disease/s |
| OR | Odds Ratio |
| PAD | Peptidylarginine deiminase |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PC2 | Physical Containment Level 2 |
| PCR | Polymerase Chain Reaction |
| PDDD | Perth Demyelinating Disease Database |
| PMA | Phorbol Myristate Acetate |
| PPE | Personal Protective Equipment |
| PPMS | Primary Progressive Multiple Sclerosis |
| RA | Rheumatoid Arthritis |
| RC | Red Cross |
| RNA | Ribose Nucleic Acid |
| ROC | Receiver Operating Characteristic |
| RP-HPLC | Reversed-Phase High-Performance Liquid Chromatography |
| RRMS | Relapsing-Remitting Multiple Sclerosis |
| SLE | Systemic Lupus Erythematosus |
| SNP | Single Nucleotide Polymorphism |
| SPMS | Secondary Progressive Multiple Sclerosis |

| | |
|---|---|
| SST | Serum Separating Tubes |
| STP | Streptavidin |
| TAP | Transporter Antigen Processing |
| TBS | Tris-Buffered Saline |
| TIFF | Tagged Image File Format |
| TMB | Tetramethylbenzidine |
| TNF | Tumour Necrosis Factor |
| UMRN | Universal Medical Record Number |
| UVR | Ultra-Violet Radiation |
| VCA | Viral Capsid Antigen |
| VDR | Vitamin D Responder |
| vIL | Viral Interleukin |
| VZV | Varicella Zoster Virus |
| WA | Western Australia |
| WHO | World Health Organization |

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# 1. Introduction

## 1.1. General Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Demyelination is the continual destruction of myelin, the insulating component surrounding neurons. This destruction, and associated inflammation, reduces the ability of neurons to conduct impulses and thus can reduce the ability of motor skills, muscle strength, coordination, speech and vision. MS affects 1 in 1,000 individuals, making it the most common neurological disorder in young adults. In the period before modern brain-imaging techniques were developed, diagnosis was based on clinical symptomatic criteria but could only be formally ascertained during autopsy, where lesions or plaques (*'scleroses'*) were found in the brain and spinal cord. With the introduction of magnetic resonance imaging (MRI) in the 1980s (1), a diagnosis of MS which was initially only prompted by the recognition of clinical neurological symptoms, could now be confirmed with demonstrated dissemination of inflammatory brain and/or spinal cord lesions in space (DIS) and time (DIT) (2). Currently, most treatments target suppression of the inflammatory environment within a patient, but studies are continuing to understand what combination of factors are necessary and/or sufficient to cause MS. Genetics and environmental factors are the two largest confirmed contributors to MS risk, with continued investigation into potential biomarkers aiming to elucidate the mechanisms that underpin epidemiological risk factors of MS development and progression. A key challenge for investigating this debilitating disease is that research is trying to find the cure and cause of the disease simultaneously, so that the success of treatment interventions can inform research into MS pathogenesis, which can in turn inform new therapeutic strategies. Decades of research have shown that the disease does not have a single causal factor and that risk factors for MS disease onset may not necessarily influence subsequent disease progression, so treatments must be established to target all stages of the disease spectrum in order to aid patient quality of life.

## 1.2. Impact of MS on global health

### 1.2.1. Global prevalence of MS

According to the MS International Federation in 2013 (3), MS affects 2.3 million people worldwide, with over 23,000 diagnosed people living in Australia. The number of diagnosed individuals has increased over the past decades (4), although this could potentially be attributed to increased expertise in diagnostics rather than legitimate increase of disease prevalence. For a study by the World Health Organization (WHO), worldwide prevalence was reviewed from data collected between the 1970s to mid-1990s (5), although prevalence has increased since this publication. The WHO report showed that comparison of different studies needs to address population variability (such as age and ethnic composition (6)), determination of benign/early cases (7), medical and diagnostic access and knowledge (7, 8), and different diagnostic criteria as well as inter-observer variability (6). A more recent collaboration organized by the MSIF estimated the world-wide prevalence at 33/100,000 (3) and provided data for differences between countries, summarized in Figure 1-1. Regionally, median estimated prevalence of MS is greatest in Europe and North America (108 and 140 per 100,000 respectively) and the lowest in East Asia and Sub-Saharan Africa (2.2 and 2.1 per 100,000 respectively). The MSIF reported average onset at 30 years old and a global 2:1 female to male ratio, while other publications report a higher ratio of 3.5:1 (9). The 2013 MSIF study did not report global incidence (new diagnoses), due to limited data contribution from some countries. Estimated incidence is greater in high income countries, and proportionally lower for decreasing income, which could be due to limited access to diagnostic facilities such as MRI but may also reflect true differences in disease risk. A strong geographical pattern can be identified: MS is more frequent in countries further away from the equator, in both hemispheres.

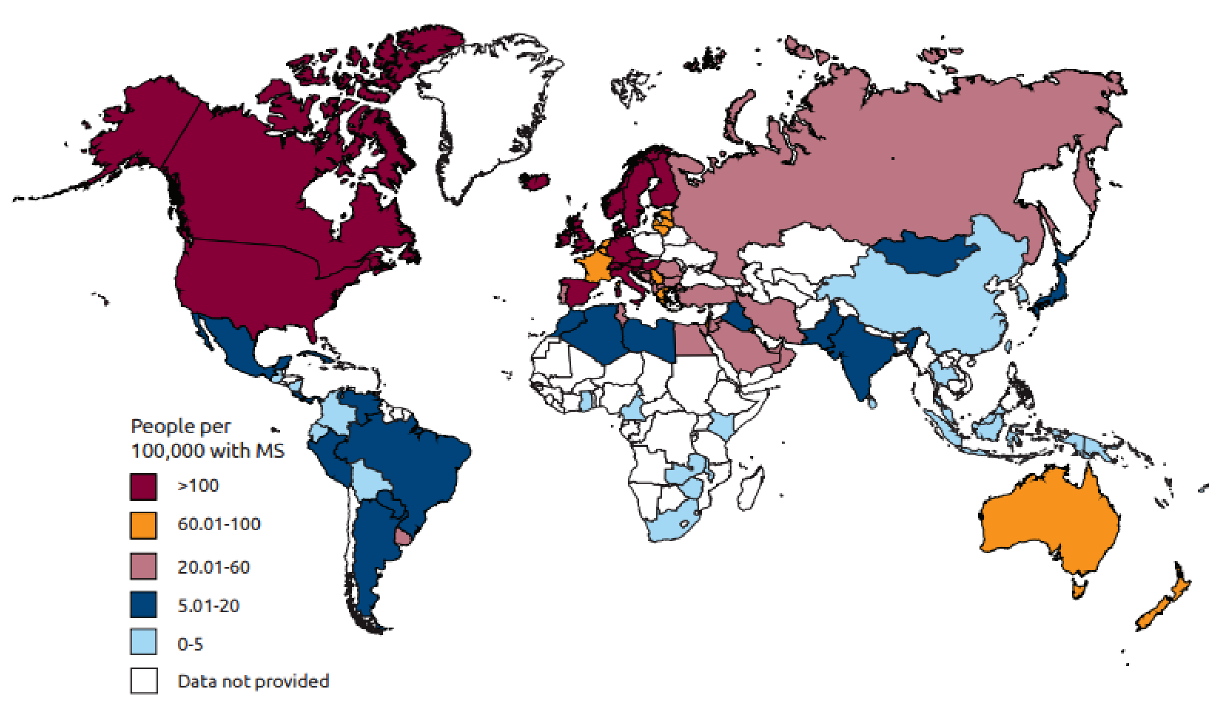


**Figure 1-1. Worldwide prevalence of Multiple Sclerosis (MS).** Higher MS prevalence can be seen in North America and Europe, as well as a latitudinal gradient with increased MS prevalence further away from the equator. Figure unedited from (3).

### 1.2.2. Prevalence of MS in Australia

Australia is a large continent within the southern hemisphere, and spans multiple latitudes. There is limited current reporting on Australian MS prevalence, but in a seminal study from 1968, McCall *et al* (10) reported prevalence rates from capital cities across the country, where Brisbane, Queensland (27.468°S, 153.028°E) had a lower prevalence than Hobart, Tasmania (42.881°S, 147.325°E). Perth, Western Australia (31.952°S, 115.859°E) had a moderate rate of prevalence (20/100,000) and a 2.3:1 ratio of females to males. This report greatly under-represents current prevalence, as do subsequent reports (11-13) which used data from 1981. Others have studied regional areas of Australia such as New South Wales and South Australia (14), as well as the city of Newcastle (15). Interestingly, these papers did not report MS cases among individuals who identified as Aboriginal or Torres Strait Islander (which was reported as “rare” by Miller *et al* (11)). The most recent publication of Australian MS prevalence is part

of the ATLAS study (a collaboration of MSIF and WHO) (3), but only at a national level, not separated by state or latitude. Current higher prevalence could be due to better and earlier diagnosis (11). The Australia Bureau of Statistics stated in a 2009 survey (16) that there was an estimated 23,700 Australians with MS (0.1% of the population), of whom 11,400 (48%) had profound or severe core-activity limitation. An estimated three quarters of these individuals were female. Of the 20,400 people aged 15-64 who were living with MS, 9,800 were employed, with 5,900 part-time, and 12,700 (62.5%) of individuals had some employment restriction (needing time off work, restricted in job type, restricted in hours, difficulty changing jobs and/or needs additional support). In addition, the survey reported that 15,800 (66.7%) needed assistance with at least one of the 10 everyday activities, of which the most common was mobility (reported by 46% of MS individuals). MS was the known cause of a "very small" number of deaths (56 male/106 female) in 2013 and was reported as an associated cause of a further 266 deaths in that year (84 male/182 female) (17).

## 1.3. History, diagnosis, lesions and treatment of MS

### 1.3.1. History of MS

There is a range of historical personal accounts (18) of what is now thought to be MS, based on descriptions of clinical symptoms (Figure 1-2). Anatomically, MS was defined by Dr Jean-Martin Charcot in 1877, from post-mortem brain lesions of a female patient who had presented an unusual combination of neurological symptoms. The term MS originates from the French 'sclerose en plaques' meaning multiple lesions or plaques. This finding added a tangible link to presented symptoms, and in 1916 James Dawson identified microscopic evidence of inflammation and demyelination. It wasn't until 1947 that researchers identified proteins ('oligoclonal bands', OCB) in patients' cerebrospinal fluid (CSF), and in the 1970s genetic associations were identified. By this time, treatment of MS already utilized anti-inflammatory

steroid treatments. Since then, advances in technology enabled further findings of MS-specific pathologies, and these continuously assist in developing new targeted immunotherapies.

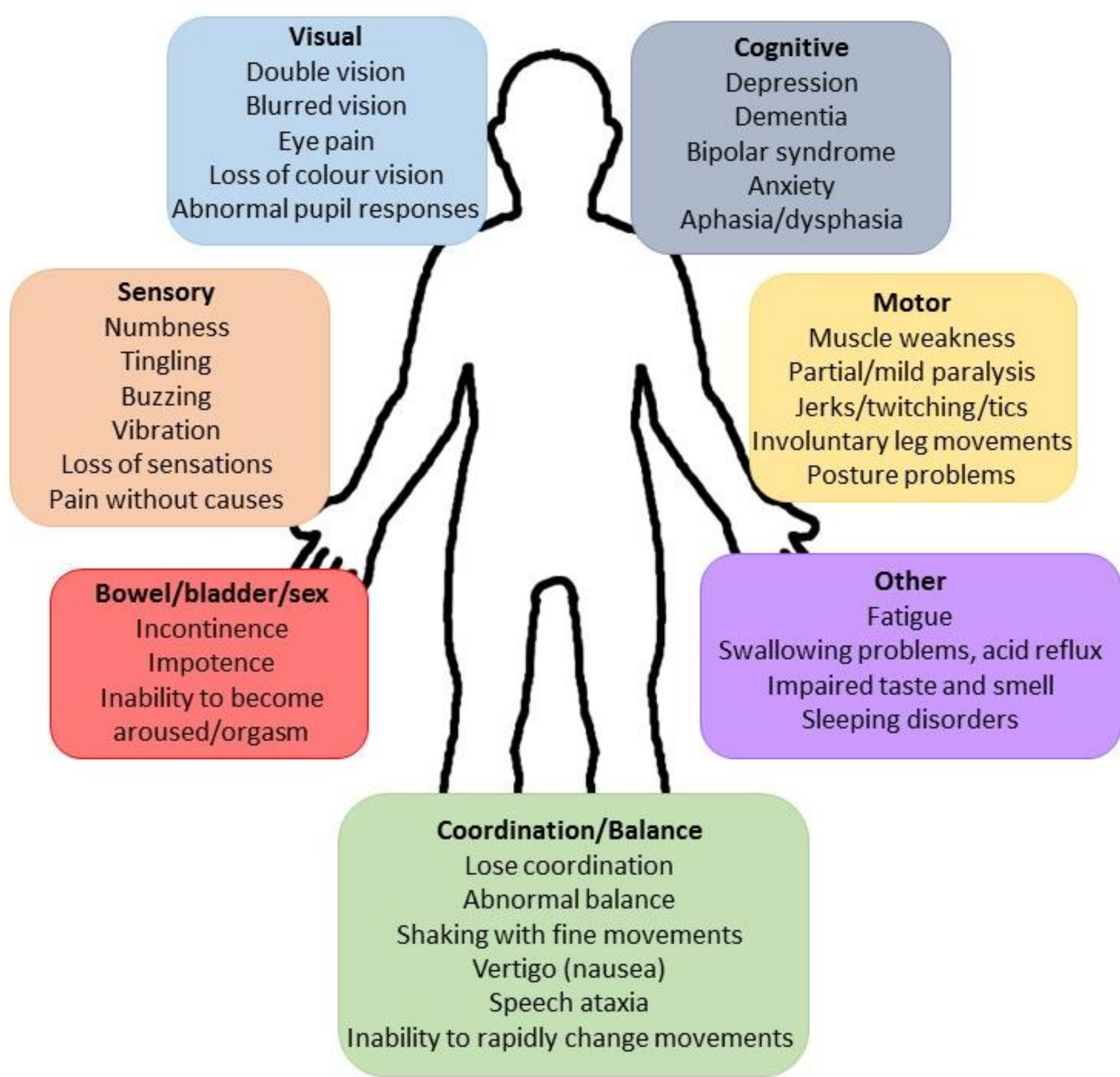


**Figure 1-2. Clinical symptoms common for suggested Multiple Sclerosis.** Wide variation of symptoms can occur between individuals as well as for a patient over time.

### 1.3.2. Diagnosis of MS

Diagnosis of MS is based upon dissemination of lesions in time and space, combining patient history, clinical examination and laboratory findings. These diagnostic criteria have been developed over the past 50 years, starting when the Schumacher Committee published the first official criteria in 1965 (19). This was before knowledge of OCB or MRI, and so was based upon fulfilment of the criteria below (i-vi), from which individuals were categorized as either having 'clinical' MS, 'probable' MS or 'possible' MS:

i. Presentation of neurologic exam abnormalities, suggesting problem in the CNS.
ii. Onset of symptoms in patient between 10-50 years of age
iii. Signs and symptoms indicating CNS white matter involvement and/or damage
iv. Evidence of two or more areas of CNS involvement, suggesting lesion dissemination in space (two or more separate lesions)
v. Either two attacks/relapses lasting ≥24 hours at least one month apart *or* symptom progression (slow or stepwise), suggesting lesions are disseminated in time
vi. No better explanation

This has served as a template for all further development of diagnosis criteria. Poser *et al.* (20) formed a set of criteria as a guideline for research protocols which was later adopted in clinical practice, that included laboratory evidence in the form of OCB Immunoglobulin gamma (IgG) production in CSF (Table 1-1).

**Table 1-1. Summary of Poser Criteria for Multiple Sclerosis Diagnosis.**

| | Attacks | Clinical lesions | | Laboratory or paraclinical lesions | CSF Bands or IgG |
|---|---|---|---|---|---|
| **Clinically definite MS** | 2 | 2 | | | |
| | 2 | 1 | and | 1 | |
| **Laboratory-supported definite MS** | 2 | 1 | or | 1 | + |
| | 1 | 2 | | | + |
| | 1 | 1 | and | 1 | + |
| **Clinically probable MS** | 2 | 1 | | | |
| | 1 | 2 | | | |
| | 1 | 1 | and | 1 | |
| **Laboratory-supported probable MS** | 2 | | | | + |

Table adapted from (21).

Using the Poser criteria, patients were defined into two groups: 'definite' or 'probable' MS, both with subgroups 'clinically supported' and 'laboratory supported', with further analysis of evoked potentials and spinal fluid evaluations to document asymptomatic damage in the CNS, confirming dissemination in space and time. Clinically definite MS required occurrence of two separate attacks (relapses) and clinical evidence of two separate lesions, or two attacks with clinical evidence or one lesion and paraclinical evidence of another separate lesion. The McDonald criteria were published in 2001 by The International Panel on the Diagnosis of Multiple Sclerosis, integrating MRI assessment to identify lesions, as well as diagnosing new or active lesions by Gadolinium (Gd)-enhancing examinations as well as visual evoked potentials and CSF analysis (Figure 1-3). They also reviewed the diagnosis of primary progressive MS (PPMS), although this was further classified years later (22, 23). McDonald and Poser criteria were compared by Fangereau *et al.* (24), who found that inclusion of

laboratory evidence was beneficial in diagnosis, and that the McDonald criteria enabled diagnosis after a single attack or relapse (an exacerbation or presentation of new symptoms), allowing earlier diagnosis and initiation of treatment. In 2005, a revision of the McDonald criteria was released (25), demonstrating dissemination of lesions in time, clarifying the use of spinal cord lesions and allowing for a simplified approach to diagnosis of PPMS. However, these criteria were suggested to be unsuitable for paediatric cases, and are being reviewed for their application by the Asian Neurological Community and in Latin America (2). Difficulties in MS diagnosis were also reported by Confavreux *et al* (26), stating that only about one tenth of new/active lesions result in a clinical relapse, depending on location and volume (27). Uitdehaag *et al.* (28) have proposed standardizing the approach to interpretation of clinical symptoms and signs of the first episode of neurological symptoms, 'Clinically Isolated Syndromes' (CIS) and Swanton *et al.* (29) have also advocated for simplified MRI criteria for DIT and DIS for relapse-remitting MS (RRMS). The most recent review in 2011 by Polman *et al* (2) has again revised the McDonald criteria, and a current summary of the updated criteria (Table 1-2) has been made available by the International Panel for Diagnosis of MS.

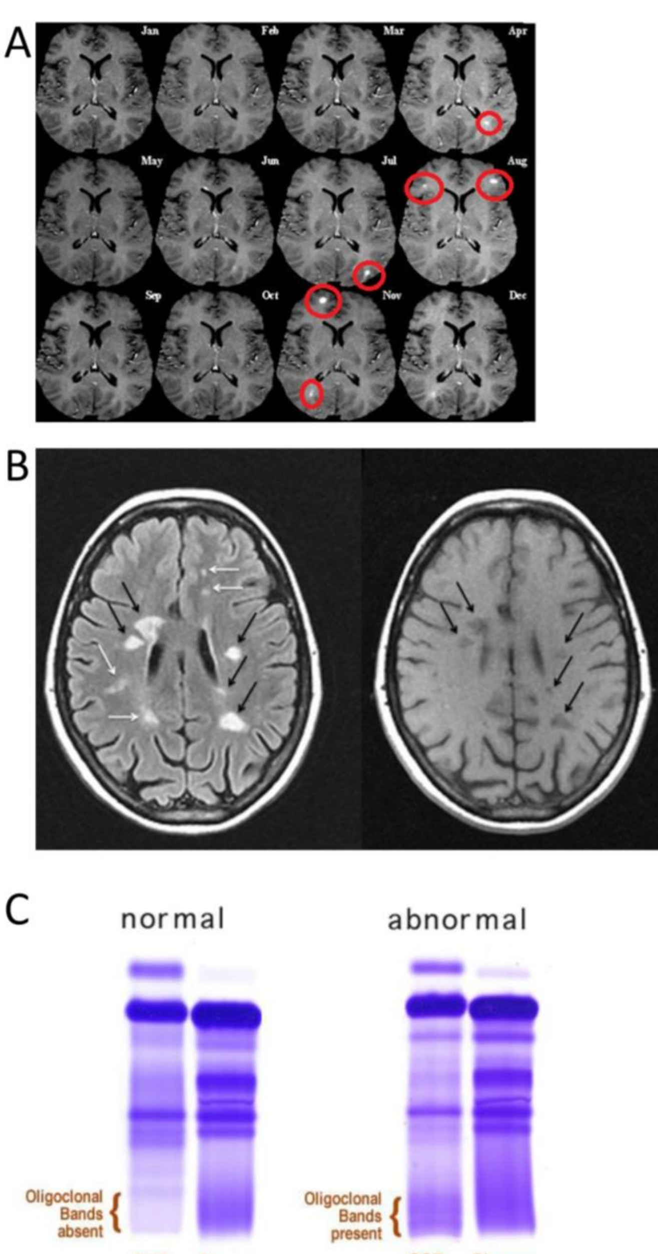


**Figure 1-3. Three laboratory techniques for MS diagnosis.** Following presentation of clinical symptoms, MS lesions can be identified by (A) Standard Magnetic Resonance Imaging (MRI) with Gadolinium enhancement (30) as well as (B) T2-weighted fluid-attenuated inversion recovery (FLAIR; left; lesions) and T1-weighted (right; holes). Black arrows indicate older lesions with more damage (as matched in the scan on the right), while white arrows identify new lesions (31). Additionally, (C) Oligoclonal bands can be identified in CSF fluid of MS patients, unedited from (27).

**Table 1-2. Revised McDonald MS Diagnostic Criteria (2011).**

| Clinical attacks | Lesions | Additional Criteria for Diagnosis |
|---|---|---|
| **Two or more** | Two or more | None |
| **Two or more** | One | DIS, demonstrated by:<br>• MRI *or*<br>• 2 or more MRI lesions plus positive CSF<br><br>or await further clinical attack implicating different CNS site |
| **One** | Two or more | DIT demonstrated by<br>• MRI<br>• Second clinical attack |
| **One** | One | DIS or await further clinical attack implicating different CNS site AND DIT; or await second clinical attack |
| **Zero** | | One year of disease progression (retrospective or prospective) *and*<br><br>Two of the following:<br>• Positive brain MRI<br>• Positive spinal cord MRI<br>• Positive CSF |

DIS: Dissemination in space. DIT: Dissemination in Time. MRI: Magnetic Resonance Imaging. CNS: central nervous system. CSF: cerebrospinal fluid.

Globally, MSIF (3) reported that 96% of surveyed countries use the McDonald criteria, with the remaining 4% assumed Poser and/or Schumacher criteria. The discrepancy in criteria could be due to access to diagnostic techniques, such as MRI machines. It should be noted that this also doesn't take into account individual neurologist's diagnoses, but is currently the best means of diagnosis until a singular diagnostic test is developed.

Initial symptoms of CIS may depend on the sites of demyelination, and can vary between patients (Figure 1-2). The three most typical presentations are:

i. Optic nerve (optic neuritits) e.g. Blurred vision

ii. Spinal cord (transverse myelitis) e.g. Tingling, prickling sensation in skin

iii. Brainstem (brainstem syndrome) e.g. Nausea, vomiting, double vision.

Patients with CIS have a high likelihood of developing MS. Exacerbations (relapse, attack, flare up), are defined as episodes of clinical worsening with either new symptoms or the worsening of old symptoms. Following diagnosis of MS, there are different ways the disease can progress.

### 1.3.3. Classification of MS progression

MS has until recently been classified into four types of disease progression (32), outlined in Figure 1-4A. Approximately 85% of individuals develop RRMS, characterised by partial or total remission followed by another relapse (33). It has been estimated that up to 90% of RRMS patients developed secondary progressive MS (SPMS) after 25 years (34), although most publications conservatively estimate 50% RRMS convert to SPMS (35), where initial relapse-remitting course is followed by increased progression with only minor or no remissions. PPMS occurs in 15% of patients and is defined by disease progression from onset with little or no recovery from symptoms. This form of the disease is usually classified after a minimum of one year's disease progression with laboratory findings (lesions in brain and spinal cord identified by MRI and CSF abnormalities (36)), and is more commonly seen in males and older patients (33). The fourth and least common type is progressive relapsing MS (PRMS), with progressive disease from onset, with clear acute relapse, with or without full recovery (32). The most recent review defining MS clinical course by 'The MS Phenotype Group' suggested 'dropping' the term relapsing-progressive due to being vague and overlapping with other courses (37). This

group also commented on the re-examination of MS disease phenotypes, with focus on assessment of disease activity by clinical assessment of relapse occurrence or lesion activity detected by CNS imaging. This is important also with radiologically isolated syndrome (RIS) where inflammation is detected but no symptoms manifest (37). A somewhat controversial diagnosis is 'benign' MS, where a patient following initial CIS does not suffer relapse attacks, or progressive worsening of symptoms for prolonged periods of time. The classification of benign MS is still being reviewed, as some studies found that at most 55% of initially 'benign' diagnosed patients remain benign over the subsequent 10 years (38, 39). Pathologically, more lesions have been found in SPMS compared to PPMS (40, 41). However, MRI activity appears to decrease with time and age, and Ebers (32) found no significant difference between the two progression types. It is important to note that classification of a patient into one or the other category usually can only be made after several years, with tracking of worsening disability, which can be graded by Expanded Disability Status Scale (EDSS; Figure 1-4B).

Early MS diagnosis is important for patients as immunomodulatory therapy introduced earlier is beneficial and has shown prolonged time to subsequent relapses, reduced MRI lesions and slower progression (42-44). There has not been much success with identifying risk factors in early MS disease that can predict how MS will progress for individual patients. However, Debouverie *et al* (45) summarized that numerous studies that identified groups with later onset of long-term irreversible disability: females, younger patients, patients with an initial relapse-remitting course, those with complete recovery from first neurological episode, patients with symptoms reflecting one region rather than poly-regional lesions, individuals with a low number of relapses during the first years of the disease and patients with longer periods of time between the first two attacks (26, 32, 46-51). However, none of these variables remained predictive of the time course of disability after the point of initial assignment of irreversible

disability, indicating that when a detectable threshold of irreversible disability has been reached, the disease enters a final common pathway (26).

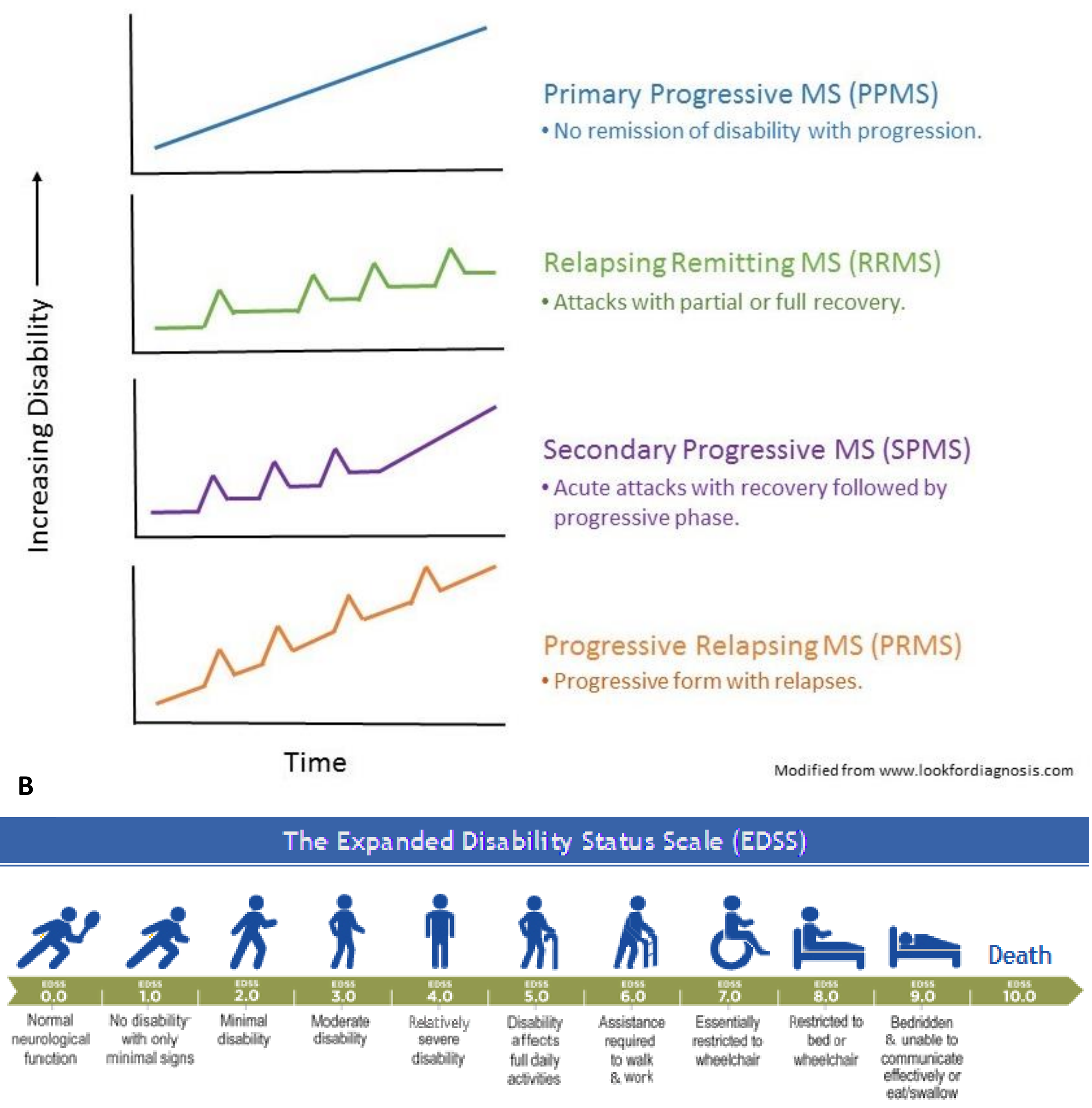


**Figure 1-4. (A) Four classifications of MS progression and (B) Kurtzke's Expanded Disability Status Scale (EDSS).** (A) The four main types of MS is defined by presence of remission between relapses (exacerbation or new symptoms) as shown by peaks. The solid line shows no remission from disease progression. (B) A scale outlining progression of motor skill disability, often used to identify worsening of symptoms, figure unedited from (52).

### 1.3.4. Current MS medications

Disease modifying treatments were first introduced in 1993 with interferon beta-1b (53), and to date there are at least eight FDA-approved treatments to reduce MS symptoms and prolong time between relapses. Most treatments are aimed at RRMS cases, as treatment for progressive patients has not been shown to be as effective. However, there is no medication to date which is universally effective for MS patients. Treatment can be grouped into two main categories: treatment for easing individual symptoms, and treatment which is disease modifying with immunomodulatory/anti-inflammatory activity. As MS is considered an autoimmune disease, with inflammation seen in brain lesions, immunomodulatory and immunosuppressive drugs are the current standard for treatment (Table 1-3). Monitoring patient progression and inflammatory activity is vital, as new lesions can be identified with MRI, and change of treatment might be indicated. A recent review by Minagar (54) outlined the success of individual treatments, and the problems associated; ranging from side effects to complications with compromised immune systems allowing opportunistic infections. Steroids including methylprednisolone, dexamethasone and prednisolone are common treatments, sometimes in addition to other medications. Not included in Table 1-3 is Cladribine, an immunosuppressant which although effective in supressing DNA synthesis and repair, was withdrawn from the European market and is awaiting follow up in the United States due to concerns about prolonged suppression of the immune system and potential risk for cancer (54). Lastly, clinical trials have begun to identify the potential benefit of vitamin D as a treatment (55), and its analogue Alfacalcidol (56). Immunomodulatory therapies are currently ineffective in modifying disease for PPMS patients (57), and treatment strategies need to be developed. There are many therapies currently under trial, including ATX-MS-1467 (58). This drug was based upon the vaccination theory, which aims to develop T cell tolerance for myelin basic protein (MBP) peptides which are thought to be a major target for inflammatory myelin-specific T cells.

**Table 1-3. Current treatments and medications for MS.**

| Treatment Type | Brand names | Mechanism of Effect | Clinical Trials |
|---|---|---|---|
| Interferon beta-1b | Betaseron, Extavia | Anti-inflammatory | (44, 59-65) |
| Interferon beta-1a | Rebif, Avonex | | (66-70) |
| Glatiramer acetate | Copaxone | Unknown, possible MHCII binding competitor | (71-75) |
| Mitoxantrone[a] | Novantrone | Immunosuppressive, Immunomodulatory | (76-81) |
| Natalizumab[a] | Tysabri | Stop leukocyte adhesion to BBB | (82-86) |
| Fingolimod[a] | Gilenya | Modulates chemoattractive function of lymphoid cells | (87-89) |
| Alemtuzumab | Campath-1H | Depletes CD52 cells (including T lymphocytes) | (90-92) |
| Teriflunomide | Aubagio | Suppresses pyrimidine synthesis (cell proliferation) | (93, 94) |
| Dimethyl fumarate | Tecfidera | Immunomodulatory | (95-99) |
| Laquinimod | Nerventra | Immunomodulatory | (100) |
| Daclizumab | Zenapax | Depletes CD25+ activated T and B lymphocytes | (101-103) |
| Methotrexate | | Immunosuppressive | (104, 105) |
| CD20+ monoclonal antibody | Rituximab | Depletes CD20+ B lymphocytes | (106) |

BBB: blood brain barrier. MHC: Major Histocompatibility Complex. CD: cluster of differentiation. (a) Immunomodulatory effect.

## 1.4. Identified risk factors of MS

### 1.4.1. Gender

Over the past decades the female to male ratio of diagnosed individuals with MS has increased, with publications reporting a ratio of between 2.3 to 3.5:1 (9, 107-111), and Pennell *et al* (112) reported that additionally males had later age of onset and more rapid disease progression. Some papers have reported that females have better prognosis than males (113) and are more likely to follow a benign disease course (114). There have been reviews of differences in MS disease associated with gender: influence on clinical course, severity prognosis, MS pathology, immunological and genetic findings in MS, response to immunotherapy, effects of gonadal hormones, the menstrual cycle and pregnancy effects on MS (112). However, the most comprehensive review of potential explanations for the gender discrepancy to date is by Greer *et al* (35). They suggested contributing factors including: biological differences of the immune system and CNS, genetic and epigenetic factors including sexual dimorphism, the effect of X and Y chromosomes, mitochondrial inheritance, microRNAs, different environments and lifestyle as well as maternal microchimerism (the mother carrying genetically distinct cells different from herself, such as a foetus). Additionally, it was reported that the predominant MS genetic risk marker human leukocyte antigen (HLA)-DRB1*1501 is more prevalent in female patients (115). If there is a female predisposition for MS, gender has to be taken into consideration when interpreting research results and for developing new therapies and clinical trials. Oestrogen is associated with MS risk at a genetic level (116), and has been shown to reduce severity and symptoms in mice models (117). The role of pregnancy in modulating MS risk is also a subject of extensive current research, as it is associated with fewer relapses (118), possibly due to a skewing of the immune system towards immunological tolerance towards the hemi-allogeneic foetus.

### 1.4.2. Genetics

The influence of genetics on MS development was initially identified through studies of-familial aggregation of diagnosed individuals, and MS prevalence in the general population has been estimated at 0.1%. Multiple cross-sectional studies of MS patients and their families (94, 119), twin studies (120-129), adoptive relatives (130), half siblings (131), conjugal pairs and their offspring (132, 133) determined a significant genetic contribution to MS risk. Additional findings included that stepchildren have the same risk as the general population (134), maternal half siblings had greater risk than paternal half siblings (131, 135) and that SPMS is more common in families with multiple affected relatives (136). Ebers *et al* (137) reported a 20-fold increase in disease risk associated with having a first degree relative with MS. A meta-analysis of 18 studies (138) reported risk of 18.2% for monozygotic twins and 2.7% for siblings, supporting genetic contribution to MS risk, of which the 57 known MS loci found in the genome-wide association study (GWAS), at the time of this publication, were estimated to contribute 18-24% of sibling relative risk. The most prominent gene association with MS risk is human leukocyte antigen (HLA) allele variation (139-141) (Figure 1-5). The HLA lies within the major histocompatibility complex (MHC), a series of highly polymorphic genes responsible for presenting antigenic peptides to immune cells, and is critical in developing the adaptive immune response to foreign pathogens (142). These genes are classified as 'class I' ("A, B and C"; present on all nucleated cells and recognised by cluster of differentiation (CD)8+ T cells) and 'class II' ("DR, DP, DQ"; present on dendritic cells, B cells, mononuclear phagocytes, some endothelial cells, epithelium of thymus and recognised by CD4+ T cells).

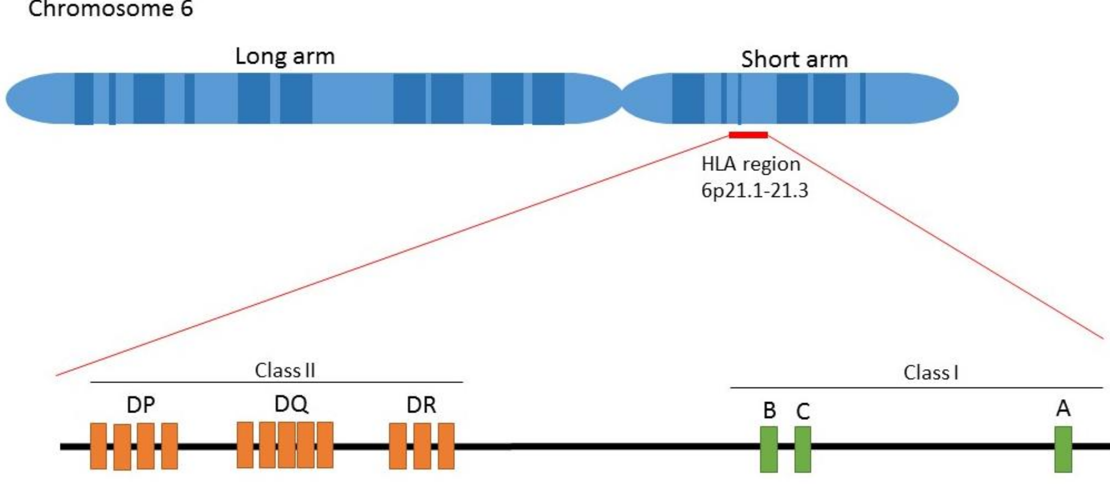


**Figure 1-5. Positioning of Human Leukocyte antigen (HLA) alleles (class I and class II) along chromosome 6.**

Advances in technology have enabled improved discrimination of HLA alleles which were initially defined by serological typing of 'class II' DR1-18 and DQ1-9. Current typing methods allow definition of at least 71 DRB alleles, 10 DQA alleles and 21 DQB alleles to four-digit resolution; that is, to the level of non-synonymous polymorphism at antigen-binding sites (exons 2 and 3) of the HLA molecule (143). For example, serotype DR15 can be sub-classified further to DRB1*15:01, *15:02, *15:03, *15:04, *15:05 or *15:07, thus allowing deeper investigation of risk alleles for MS, and understanding of their functional abilities. A genome-wide linkage study in affected relative pairs (144) identified HLA as a MS risk factor, but numerous studies have identified other genetic risk factors, as summarized in Table 1-4. The varying frequency of HLA alleles in ethnically different populations has led to different prominent risk alleles within these populations, and has contributed to population-specific differences in MS prevalence overall. At the haplotype level, serotype DR2 is common in Caucasian MS populations (145-147), while DR3 and DR4 are common in Sardinians (148-150), DR4 in Jordan (151), Canaries (152), Turkey (153) and Spain (154), while DR6 is prevalent in Japan (155) and Mexico (156). With four digit HLA-typing, the dominant

association for Caucasians (157) (Table 1-5) or ‘Europeans’ in Australia (158, 159) is HLA-DRB1*15:01, found in over 50% of MS cases with further evidence for preferential transmission through the maternal line (160). This allele is less common in African-Americans (161) and not reported at all in Asian-type MS (162) or Israelis (163). In comparison, some alleles have been identified to have a protective effect on MS risk, including HLA-A*02 (164-166), HLA-DRB1*11 (167), -DRB1*12 (150, 168, 169), -DRB*14 (170, 171), -DRB1*07 (150), as well as -DRB1*04 and -DRB1*01, but only in combination with -DRB*15 (158). When comparing high risk and low risk heterogeneity, a dose-dependent effect of HLA has suggested multiple genetic contributions (169). Studies of linkage disequilibrium (genes inherited grouped together rather than completely independently) and epistasis (the effect a gene has on other genes, directly or indirectly) resulted in further refinement. Evidence of these mechanisms includes DRB1*15 high risk being abrogated by DRB1*14 (170, 171) and HLA-A*02 (164); DQA1*0102 only contributing to MS risk in presence of DRB1*1501, (172), and doubling of DRB1*08-associated risk when present with DRB1*15 (170, 173), while DQA1*0101 and DQB1*0501 are protective with DRB1*15, but neutral in its absence (172). The combination of genes DRB1*1501-DQA1*0102-DQB1*0602 (Haplotype ‘DR15’ (174-176)) is common in Northern European MS cases (177-180), while a stronger risk is identified for DRB1*0301-DQA1*0501-DQB1*0301 (DR3) in Southern Europeans (152, 153, 181), suggesting groups of alleles have stronger risk than a single locus (177, 182, 183). This is supported by our own research (184) showing grouping of HLA-DRB1 alleles as “high risk” and “low risk” stratified MS risk in an Australian Caucasian population (Figure 1-6). In terms of linkage disequilibrium, DRB1*08 is preferentially and over-transmitted with DRB1*1501 (139, 172, 173) and DRB1*1501 and DQB1*0602 are nearly always located together, making it difficult to determine whether only one of them is the causative risk factor (172, 185). Associations between HLA alleles and disease onset, severity and progression have been

investigated, with conflicting results (Table 1-5), including an in-depth review (186). Not included in the table were additional studies reporting no genetic association with clinical course (150) or lesion development (187-190).

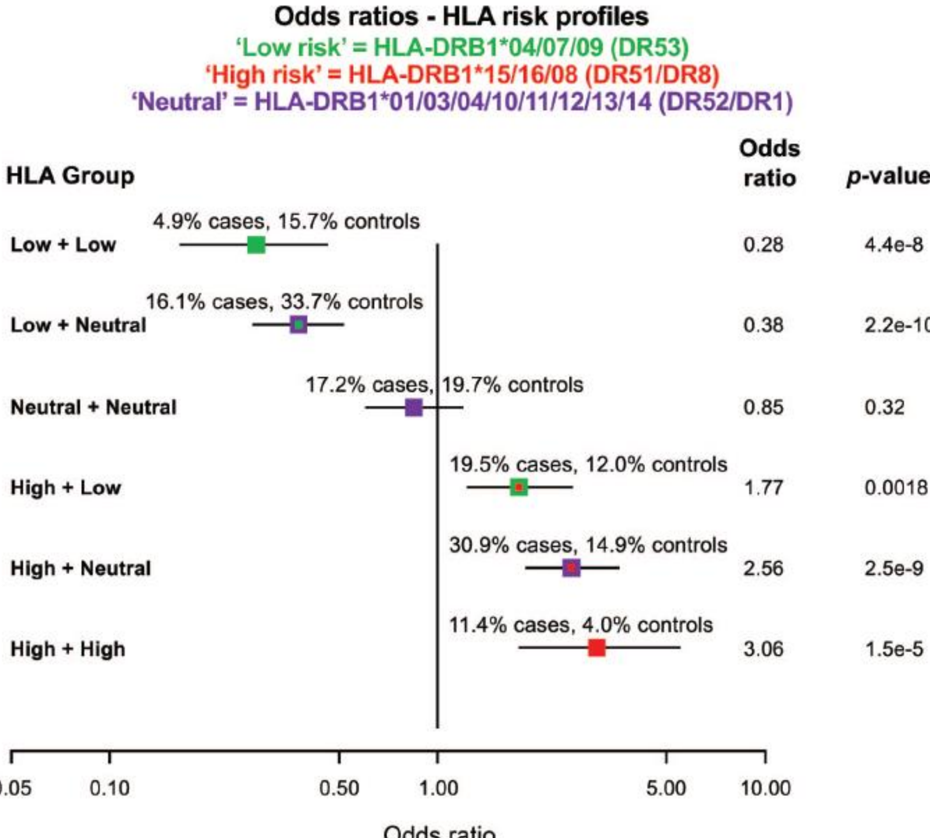


**Figure 1-6. Stratifying MS risk according to combined HLA allele profiles from 498 MS cases and 498 controls.** Boxes show odds ratio with 95% confidence intervals, and identify stratification of MS risk with absence of "low risk" HLA-DR alleles and presence of "high risk" HLA-DR alleles. Figure unedited from (184).

**Table 1-4. Summary of HLA and non-HLA genes associated with MS risk.**

| MS risk gene | Published studies |
|---|---|
| **HLA** | |
| **HLA-DR (6p21)** | (178, 191-195) |
| **DR1*15** | (150, 158, 165, 166, 168, 170-172, 185, 187, 196-198) |
| **DR17** | (168) |
| **DRB1*03** | (158) (only with 1501) (187, 199) |
| **DRB1*10** | (150) |
| **DRB1*13** | (163, 165) |
| **DR2** | (185, 200-206) |
| **DQB1*02 *03 *06** | (147, 165, 187, 202, 206-208) |
| **Non HLA** | |
| ***Cytokine Pathway*** | |
| **IL7R/IL7Rα** | (158, 165, 191, 194, 197, 209-215) |
| **IL7** | (165, 213) |
| **IL2R/IL2Rα** | (165, 182, 191, 194, 197, 214) |
| **IL1 cluster** | (216, 217) |
| **IL1 beta** | (182) |
| **CXCR5** | (165) |
| **IL12B1** | (165) |
| **IL22RA2** | (165) |
| **IL12A/ IL12B** | (165) |
| **IRF8** | (165, 218) |
| **TNFRSF1A** | (165, 218) |
| **TNFRSF14, TNFS14** | (165) |
| **SOCS1** | (213) |
| ***Co-stimulatory*** | |
| **CD6** | (218, 219) |

| | |
|---|---|
| **CD37, 40, 80, 86** | (165) |
| **CD58** | (165, 194, 197, 214, 220) |
| **CLECL1** | (165) |
| ***Signal transduction*** | |
| **CBLB, GPR65, MALTI, RGS1, STAT3, TAGAP** | (165) |
| **TYK2** | (165, 197, 213) |
| ***Environmental*** | |
| **CYP27B1 (vitamin D)** | (165) |
| **CYP24A1 (vitamin D)** | (165) |
| **VCAM1 (Natalizumab)** | (165) |
| ***Viral*** | |
| **EVI5** | (194, 214) |
| **EVI5-RPL5** | (197) |
| ***Anti-inflammatory*** | |
| **CTLA-4** | (221, 222) |
| ***Other*** | |
| **T cell receptor gene** | (223, 224) |
| **Apolipoprotein E** | (182, 223, 225-227) |
| **12q13-14** | (197) |
| **20q13** | (197) |
| **CLEC16A** | (194, 197) |
| **KIF1B** | (228) |
| **STK11 (SNP)** | (229) |
| **ASF1B** | (195) |

HLA: Human Leukocyte Antigen. IL: Interleukin.

**Table 1-5. Association of HLA alleles and disease progression and course.**

| HLA haplotype | Disease Association |
|---|---|
| **HLA-DR15** | Lower age of onset, no influence on course or outcome (168, 230) |
| | No link to more severe disease progression, clinical or MRI (223) |
| | Increased risk (231) |
| | Younger onset, no link to lesion development (115) |
| **HLA-DRB1*1501** | Normal Appearing White Matter, brain volume, 1501+ more woman and younger mean age for onset, less cognitive function (232) |
| | Younger onset, no link to clinical course (165) |
| | Multiple Sclerosis Severity Score (MSSS) increased dose-dependently (169) |
| | Presence in both PPMS and RRMS, no clinical significance (170) |
| **DRB*1201** | More severe MRI changes, but not lesion development (187) |
| **DR1** | Unfavourable outcome (233) (not confirmed (168, 206)) |
| | Decreased risk or RRMS/SPMS compared to PPMS (187) |
| | Protected against PPMS vs RRMS (158)(*01/*15) |
| | Protective on outcome when with DRB*15, but split relapsing/benign and progressive (234) |
| **DR2 (DRB1*1501-DQB1*0602)** | Development from optic neuritis, especially with baseline brain MRI abnormalities (202) |
| | Increased frequency in RRMS (235) |
| | Worse prognosis (179, 185, 236, 237) |
| | More benign (217, 238) |
| | Long term no influence (168, 233, 239) |
| | Increases risk, less benign and more severe progression (185) |
| | No correlation to 'type of disease' (240) |
| | More brain lesions (202) |
| **DR4** | Increased frequency in PPMS (201, 206, 235, 241) |
| | Protected against PPMS vs RRMS (158) |

HLA: Human Leukocyte Antigen. MRI: Magnetic Resonance Imaging. RR: Relapse remitting. PPMS: Primary Progressive MS. SPMS: Secondary Progressive MS.

Class II MHC molecules can present longer peptides, while class I are restricted to binding peptides 8-10 amino acids (aa) long (242). As HLA-DR presents antigens to CD4+ T cells (243, 244), Wu *et al.* (169) suggested that heterozygous, co-dominant HLA-DR alleles could present different antigens to the immune system, such as presenting a disease-associated epitope and acting through cytokine networks simultaneously, contributing to disease pathogenesis. The HLA-DRB1*1501 receptor also contains an ideal binding region for presenting MBP peptides shown *in vitro* (245, 246) and MS lesion-associated $CD4^{+}$ T-cell TCRs have been shown to recognize the MBP peptide (aa87-99) (247). DRB1*1501 is also linked to increased pro-inflammatory cytokine tumour necrosis factor alpha (TNF-ɑ) secretion (248) and in response to MBP (249). More research is warranted, particularly into the quantitative expression of HLA based upon cell types as well as functionality of HLA-DR alleles in context of disease pathogenesis, especially with consideration of the influence of grouped alleles.

### 1.4.3. Environment

Genetic risk factors account for only 20-30% of MS risk (124, 191, 197, 250), suggesting a major role of environmental factors. The strongest evidence of this includes smoking, latitude, adolescent obesity (251, 252), ultraviolet radiation (UVR), vitamin D levels and infectious agents. These factors could be additive as well as cumulative over the lifetime of the individual, but appear to be vital in determining the susceptibility, triggering and outcome of MS (253). Thus far, there is little evidence for a prominent role of epigenetic factors such as methylation, histone modification and non-coding RNA (128, 140, 254-256) in predisposing for MS, although this research field is an emerging area of interest. The "Hygiene hypothesis" evolved from epidemiological associations between MS and improved sanitation (257), lower sibling number (258, 259) and birth order (260). In addition to more recent studies contradicting the correlation with lower sibling number (260, 261), this theory conflicts with evidence of an

infectious influence on disease risk (259), and reports of stepchildren of MS cases remaining at baseline population level risk also argues against the influence of a common familial microenvironment (253). Smoking is more prominent in MS diagnosed individuals, and increases the risk of MS (199, 262-270), but is an easily preventable risk. Genetics alone do not explain the influence of the 'latitude gradient' on MS risk, in which incidence (risk) is 20 times higher and prevalence (cases) increases from 5-10 to 200/100,000 for populations at $59^0$N compared to the equator (250, 271). This gradient (as depicted in Figure 1-1) is also seen in the Southern Hemisphere, with increased risk of MS further south of the equator (272), but inversed in several countries including regions of Italy and northern Scandinavia (271). In Europe, age and HLA-DR adjustments did not affect the association, and HLA-DR adjustment reverses the Italian inverse gradient so that it is the same as the rest of Europe (271). However, earlier studies found conflicting strength of association following adjustment for age, sex or HLA-associated genetic risk (110, 187, 273). These gradients can also be seen in migration studies, where it has been observed that migration before 15 years of age from low to high latitude can increase MS risk (274-276), although Hammond *et al* (277) found migration up to 30 years of age still identified this change in risk. Additional studies reported MS risk increased in the second generation following moving further away from the equator (13, 278, 279).

The latitude gradient is strongly suggested to reflect the importance of ultraviolet radiation (UVR) exposure, and the role of vitamin D in disease pathogenesis (reviewed by (250, 267)). UVR is vital in initial steps of production of 25-hydroxyvitamin D3 [25(OH)$D_3$], the measurable circulating form of vitamin D3 (280), which can locally (in kidney and other tissues) be converted to 1,25-dihydroxyvitamin D3 (1,25$(OH)_2D_3$) (267, 281). This biologically active hormone can bind to the Vitamin D Receptor (VDR) which acts as a promoter for transcription (282). There have been recent studies suggesting that VDR-dependent signalling

could influence HLA-DR1*1501 expression and subsequent antigen presentation with deletion of autoreactive T cells (196). UVR is a dominant source for vitamin D (283) and during winter in higher latitudes UVR is absorbed by the atmosphere, reducing exposure (284). Additionally, vitamin D deficiency is common in higher latitudes (285-289). MS risk, prevalence and mortality have been associated with low UVR or "sunshine exposure" (199, 253, 290-297) and vitamin D deficiency (267, 295, 298-301) while high 25(OH)$D_3$ levels are associated with slowed progression (302). Low vitamin D levels have been suggested to facilitate activation of autoreactive T cells, promoting a pro-inflammatory environment (303). Vitamin D supplementation increases levels of 25(OH)$D_3$ (304), reduces MS risk (299) and has shown promise in clinical trials (55, 305, 306). Both UVR exposure and vitamin D have also proven protective in animal models (307-311), which may elucidate underlying mechanisms. The influence of vitamin D and UVR may explain the 'month of birth' effect, in which more MS cases have their have birthdays in spring (312-316), thereby suggesting that maternal end-of-winter vitamin D deficiency could influence the child's subsequent risk of MS (253) and highlighting the potential importance of early-life seasonal events (315, 317).

Multiple pathogens have been targeted as infectious risk factors. Parasites have been postulated to reduce MS risk and progression (318-320), with evidence that this effect may be mediated by the up-regulation of interleukin (IL)-10 and regulatory B cells (321, 322). The strongest positively correlated infectious risk factor has been human herpesvirus (HHV) infection, which is common in most populations. Christensen *et al* (323) reviewed that HHV-2, HHV-7 and HHV-8 have no association with MS. Some evidence exists for MS association with HHV-1 (324-326), HHV-3 (326-328), HHV-5 (325), and HHV-7 (329), but a majority of studies that looked at multiple herpesviruses found these to be non-significant. Evidence in support of a HHV-6 association includes DNA in plaques (324, 330-333), cellular immune responses to

HHV-6 (334), presence of HHV-6 DNA in serum (335-337), antibodies against HHV6 in serum and CSF (338-343), and increased virus-specific immune response linked to exacerbations (344, 345). Other studies have reported no significance in HHV-6 DNA levels or antibody responses (325, 346-351). The strongest evidence to date supports HHV-4 viral influence on MS development, commonly known as the Epstein-Barr Virus.

## 1.5. Epstein Barr Virus

### 1.5.1. History and genome of EBV

Epstein-Barr Virus (EBV), "*human herpesvirus 4"*, is a human gamma herpesvirus which is believed to have evolved with humans for thousands of years. It has the unique ability (compared to alpha and beta herpesviruses) to utilise the host system to replicate itself during latency (352) and immortalize virus-infected cells (353) with the potential to cause B cell lymphomas (354). EBV infects 90-95% of the population (355), generally during childhood or early adolescence where it can manifest as infectious mononucleosis (IM) in 35-50% of cases (4). EBV is a linear, double stranded DNA virus enclosed by a nucleocapsid of icosahedral structure, with an outer layer envelope with multiple binding receptors (356). Genes of EBV are differentially expressed during viral development and through alternating phases of latent and lytic infection. A schematic of the EBV genome is shown in Figure 1-7. Following primary infection, the virus remains dormant in B cells using only latent viral genes to replicate with the host DNA and evade immune recognition. There are three major strains of EBV: Guangdong strain 1 (GD1) from Chinese nasopharyngeal carcinoma (357), B95-8 from a North American IM case (358) and AG876 from a case of Burkitt's Lymphoma in West Africa (359-361). Understanding the mechanisms of the virus replication provides insight into its role in MS risk, and potential targeting therapies.

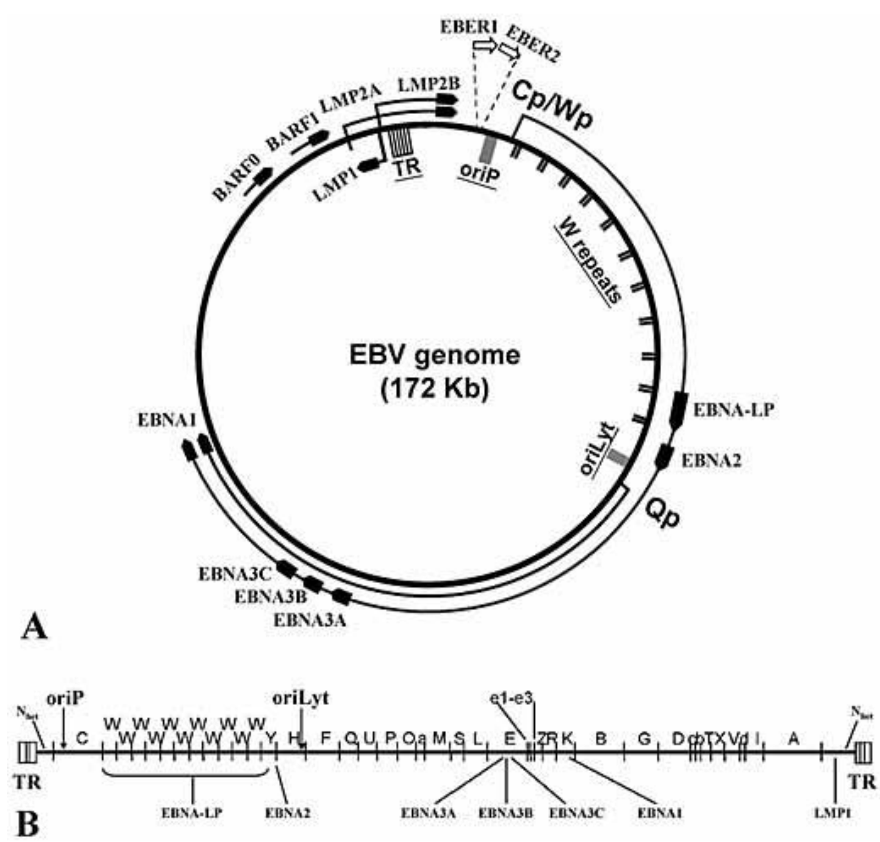


**Figure 1-7. Epstein-Barr Virus (EBV) genome (A) and open reading frame (B).** The viral genome contains genes to be expressed during latent and lytic stages of replication. Figure unedited from (362).

### 1.5.2. Life cycle

The life cycle of EBV, as depicted in Figure 1-8, can be divided into three main stages: initial infection, lytic replication with release of virions, and latency, in which the virus evades immune recognition and elimination through controlling gene products, thereby establishing lifelong persistent infection in memory B cells (363). There are five programmes controlled by EBV genes; one lytic and four latent cycles (364-367). Initial infection by EBV in naïve hosts occurs via saliva transfer (sharing food, kissing etc.), where the virus infects epithelial cells and naïve B lymphocytes in the oropharyngeal tissue (368, 369) and submucosa (370), through binding of envelope protein gp350 to the CD21 receptor on B cell surfaces (371) and second glycoprotein gp42 to HLA class II molecules as a co-stimulatory signal (372-375). Immediately

following B cell infection, EBV expresses BALF1 and BHRF1, homologues of cellular anti-apoptotic Bcl-2 protein (376). Employing latency III or the ‘growth’ programme, in which all latent viral proteins are expressed: EBV nuclear antigens (EBNA)-1, 2, 3, LP and latent membrane protein (LMP) 1, 2a and 2b (377). EBV induces proliferation of naïve B cells into lymphoblasts (366), expressing proliferative and anti-apoptotic properties (370). Lymphoblasts can be recognized and eliminated by T cells (378, 379), but latency is maintained by growth and survival signals to infected B cells (380). Lymphoblasts enter germinal centre follicles, elicit latency II or the ‘default’ programme, and become memory B cells through normal physiological maturation (364, 366, 381-383), expressing only EBNA-1, LMP1 and 2a (353, 384). LMP1 and 2a mimic signals from CD40 and the B cell receptor (BCR), respectively, enabling the B cell to mature, proliferate and survive independently of T cell help (385-393). Most importantly, only memory B cells have been identified as EBV-infected in peripheral blood so far (365, 366, 394), and infected memory B cells have been shown to evade host immunity (378). Memory cells can circulate peripherally in the latency I programme, with EBNA-1 expression within the cell nucleus (395). Latently infected B cells can return to tonsils and terminally differentiate into plasma cells, and/or initiate the lytic programme to produce and release virions from infected cells (viral “shedding”) (396) which can infect a new host (384, 397) or other cells of the same host (377). In established latency, EBV infection produces about 1-50 infected B cells per million (398). If infection occurs in early adolescence rather than childhood, IM can occur. Due to an increased number of latently infected memory B cells (399) and their elimination by the more established immune system, a massive CD8+ T cell expansion occurs to rapidly control infection (400) resulting in symptoms which can include a sore throat, swollen lymph nodes, fever and fatigue (400, 401). Following IM, viral load is usually controlled efficiently similar to other infected healthy carriers (402, 403).

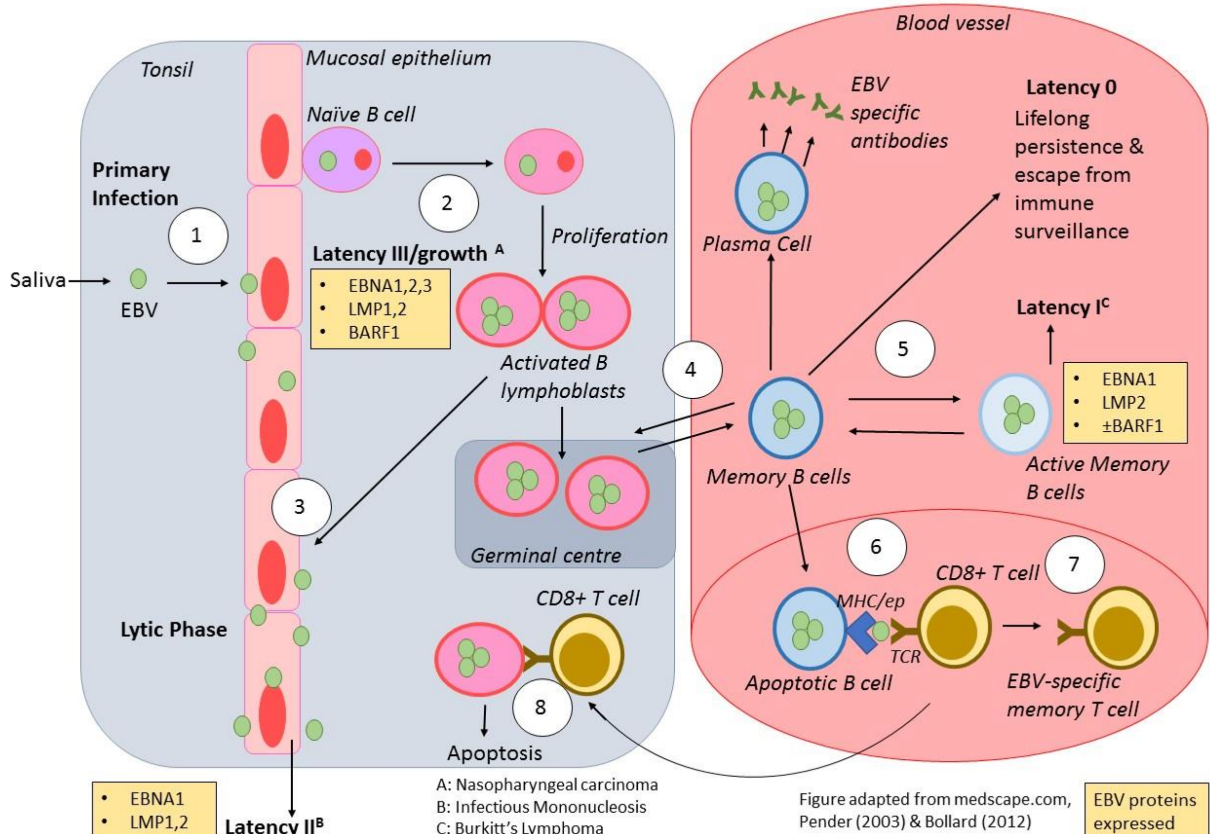


**Figure 1-8. Life cycle of Epstein-Barr Virus (EBV).** Primary infection of epithelial and naïve B cells by EBV virions (1). Viral latency III program leads to proliferation and activated B lymphoblasts (2). Release of EBV virions in the lytic phase leads to infection of new cells (3). Lymphoblasts enter germinal centres and become memory B cells (4), and enter peripheral circulation and persist in latency 0/I (5), to become plasma cells or present themselves to T cells (6). These T cells can become memory T cells (7) or move to the lymphoid tissue, eliminate EBV infected cells (8), and monitor future reactivation. Uncontrolled infection can lead to disease (A, B, C).

### 1.5.3. Immune response to EBV infection

Only quite recently has some class I HLA (HLA-C-35T/T and HLA-Bw4) been associated with EBV seronegativity (404), and single nucleotide polymorphisms (SNPs) associated with developing IM (405). As EBV infection is common in most populations, it is possible HLA may not greatly influence primary EBV infection, but could affect the immune response against the virus over the individual's life. Rubicz *et al.* (406) reviewed how HLA-DRB1 and HLA-DQB1 could potentially regulate EBV infection, as they linked to EBNA-1 IgG, but this was not seen with other viral antigens. Studies have shown the change in antibody response to

different EBV antigens over the course of infection (summarized in Figure 1-9). With initial infection, there is no cellular immunity to prevent early viral amplification (399). Immunoglobulin M (IgM) against viral capsid antigen (VCA) is identified early in infection (407), but anti-VCA IgM levels decrease by 4-6 weeks (356), switching to IgG (4) which continue to be produced for life. Anti-EBNA-1 IgG is not detectable until 3-6 weeks post-infection (4), and then persists for life. Therefore, anti-VCA IgM antibodies are most helpful to identify current infection, whereas anti-EBNA-1 IgG indicates past infection. Memory T cells can later provide protection upon viral re-exposure (408), but by the time this immunity is formed EBV has established a permanent latent infection.

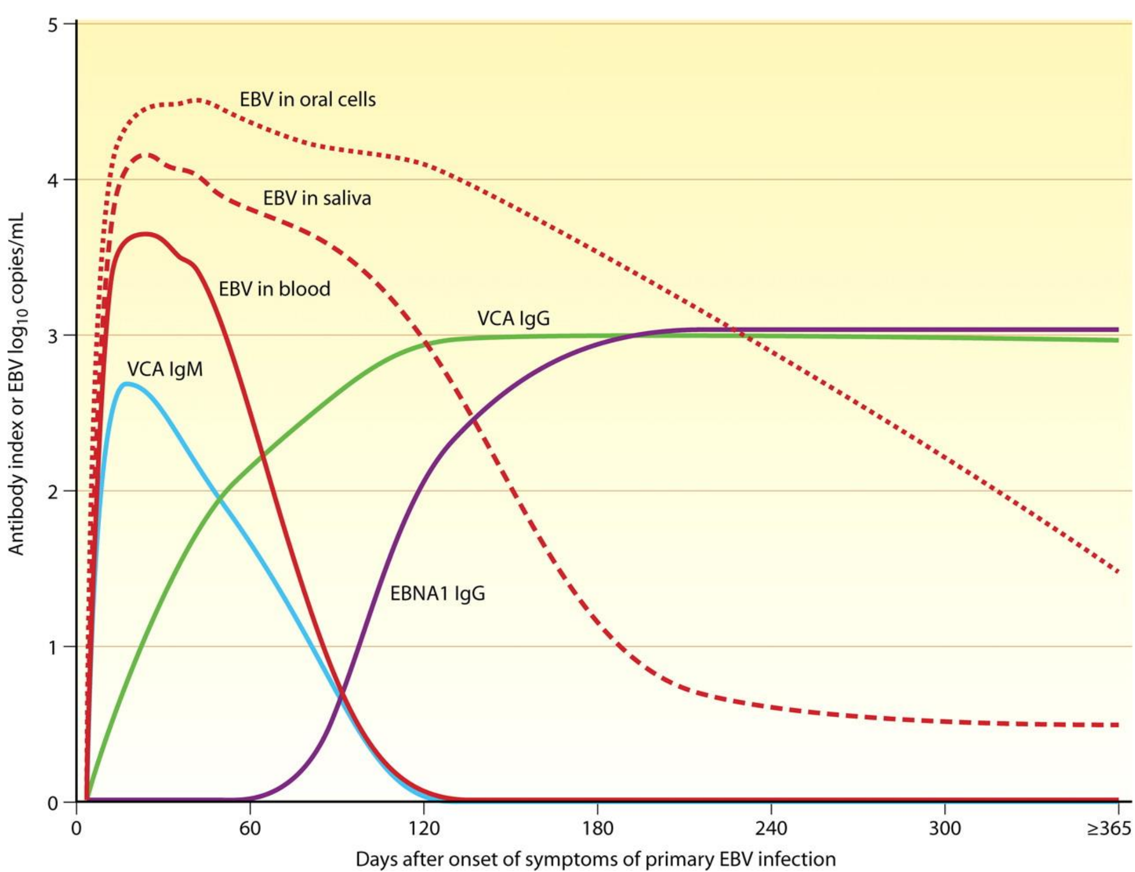


**Figure 1-9. Antibodies specific for EBV proteins after primary EBV infection.** Anti-viral capsid antigen (VCA) IgM appears early after infection and disappear by day 120 whereas anti-EBV nuclear antigen-1 (EBNA-1) IgG antibodies appear later and are clinically used as a measure of past infection. Figure unedited from (409).

### 1.5.4. Immune evasion

Through its latency program, EBV enables lifelong persistent infection, avoiding immune recognition and elimination by T cells (410, 411). Understanding these underlying viral mechanisms can be beneficial for therapy development, as it appears the viral immune evasion mechanisms reduce recognition rather than provide absolute protection from host immunity (352). Ressing *et al* (412) provided a more in-depth review of the viral genes involved in immune evasion and their mechanisms, so only selected examples are provided here. Firstly, the glycine-alanine repeat (GAr) within the middle of the EBNA-1 protein largely prevents proteasomal degradation (413-415) and thus consequently MHC-I restricted presentation to CD8+ T cells (416). However, EBNA-1 peptides from lysed B cells can be autophaged (412) and presented through MHC-II exogenously (417) to CD4+ T cells, similarly to those observed for EBNA-3 and EBNA-6 epitopes (418). Experiments utilizing lymphoblastoid cell lines (LCLs), where B cells are infected with EBV, showed GAr deletions resulting in higher presentation of EBNA-1 to CD8+ T cells, with subsequent LCL inhibition (416). Additionally, the purine bias in EBNA-1 messenger RNA (mRNA) alters its stability, enabling immune evasion (419, 420) and inhibits self-synthesis (421, 422). The genes BCRF1 and BNLF2a encode for viral interleukin-10 (vIL10), a homologue of human IL-10 (hIL-10; 423), and are expressed early in EBV infection (424). While hIL-10 can act as both immune-stimulatory and suppressive (425), vIL-10 is only immunosuppressive. vIL-10 down-regulates MHC-I and reduces mRNA expression of Transporter associated with Antigen Processing (TAP)-1 and bli/LMP2 (412, 426-430), leading to reduced processing of endogenous antigens and reduced CD8+ T cell recognition of infected cells (431, 432). vIL-10 additionally inhibits monocyte function (433) and subsequent activation of CD4+ T cells (434), cytokine response (435-437), dendritic cell maturation (438), B cell growth transformation (439) and protects B cells from natural killer (NK) cell cytotoxicity (437). Multiple EBV genes, including BILF1, BGLF5,

BZLF2 and BZLF1, down regulate MHC-I and MHC-II expression (352, 412, 437, 440, 441) with BLZF/Zta and BZLF2 in particular reducing interferon gamma (IFNɣ)-induced T cell recognition (352, 412, 442). BGLF5 is additionally involved in shutting down host protein synthesis (443) and contributes to TLR9 down-regulation in infected B cells (444). Paradoxically, LMP1 is anti-apoptotic (366), but up-regulates the antigen processing pathways (APP) to enhance T cell presentation (445). Therefore, the virus can evade immune recognition long enough to establish infection, but after this point is reached, recognition of the virus enables the host to clear lytic infection by eliminating newly infected cells, while the latently infected B memory cells can escape to the periphery.

### 1.5.5. EBV and MS

The association between MS and EBV has been demonstrated in several studies with multiple reviews on EBV as a causative agent in MS (370, 446-449). Researchers have investigated virological factors (e.g. plasma and cell-associated levels of viral proteins, antibodies against different viral proteins (including quantitative measures as well as functional characteristics)), as well as cell-mediated immune responses specific for the virus and compared findings between MS patients and controls. Originally considered as part of the poliomyelitis theory (450), which evolved to become the "Hygiene hypothesis" (as discussed in Chapter 1.4.3), a recent concept is that failed regulation of EBV infection is a potential cause of MS disease (451). In support of this, studies and meta-analyses of IM and MS risk have demonstrated an association between symptomatic early infection and MS risk (384, 452-456), and recently it was suggested MS and IM additionally share latitudinal distribution (457). Furthermore, it has been reported that the presence of HLA-DRB1*15 as well as history of IM greatly increases MS risk (458), and HLA-DRB1*15 has been shown to be a risk factor independent of high anti-EBV antibody titres (198, 459-461). The possibility that different EBV viral strains could

influence risk of MS has also been examined, acknowledging that strain prevalence differs between ethnic groups (462, 463). Strain B95-8 is most commonly used in MS *in vitro* experiments, but Lay *et al.* (464) identified GD-1 strain (genotype A) was significantly more prevalent in Australia than B95-8 (77.7% vs 16.7%). Co-infection with multiple EBV strains seems rare, and Yao *et al*. (465) reported that hosts are not re-infected with different multi-strains, over a 15 year study period. Simon *et al*. (255) found that EBNA-1 N' and C' terminus as well as LMP1 variation contributed to MS risk, and Mechelli *et al* (466) recently reported that EBNA-2 variants were also associated with MS, but not HLA or MS clinical features. No significant difference has been identified between different EBV strains and individual MS risk to date (255, 464, 467, 468).

#### *1.5.5.1. Host cellular response to EBV infection*

The first indicator of EBV association with MS was by Fraser *et al*. (469) who reported that peripheral blood mononuclear cells (PBMCs) from active MS patients had an increased tendency to spontaneously undertake EBV-induced B cell transformation *in vitro*. This observation has subsequently been replicated (470, 471). Increased frequency of EBV-infected B cells has been identified in MS CSF and peripheral blood (472). Both CD4+ and CD8+ (Cytotoxic T lymphocytes; CTL) T cells have roles in controlling EBV infection (473-475), and it is possible that failure in regulation is important in development of MS. Increased EBV-specific CD8+ and CD4+ T cells have both been identified in MS CSF (476, 477) and peripheral blood (478-480). In 2006, Lünemann *et al.* (479) identified significantly increased frequency, reactivity and IFNɣ production by EBNA-1 specific CD4+ T cells despite normal viral loads in MS patients compared to healthy controls. This significant change was not identified in T cells specific for other EBV antigens or cytomegalovirus targets. In 2008, the same group confirmed elevated IFNɣ production by EBNA-1 specific T cells in MS, and also reported EBNA-1

specific CD4+ T cells from MS patients were cross-reactive against myelin autoantigens resulting in IFNɣ production (480). In additional studies, MS CTL control of LCLs has been reported as impaired (481-483) as well as non-significantly increased (478), although it has been argued that this finding did not take into account a generalized CTL deficiency in MS (377, 484). Studies of CTL IFNɣ responses to synthesized EBV peptides have shown conflicting results with decreased (485), increased (486-488) and normal (489, 490) levels reported, for the same targets (EBNA-3, LMP, BMLF1 and BZLF1). It should be noted that endogenous EBV within LCL utilizes a patient's APP to present lytic and latent proteins, while peptide experiments bypass this pathway (377). EBV-specific immune cells have also been found to cross-react with myelin antigens (480, 491, 492), although myelin-specific lymphocytes are also present in healthy individuals to a small percentage (246, 480). More recently, Lindsey *et al.* (483) found no cross-reactivity between LCL-reactive CD4+ T cells and brain antigens. In some of these studies, memory T cells were prominent (478, 493-495), and should be considered in the scheme of immune control, but are also potentially exhausted (377, 496). Clonally expanded B cells and B cell follicles have been detected in MS brain tissue and lesions (497-504) and CSF (505-509), where memory B cells and plasmablasts have also been identified (510). T cells have also been identified in MS brains (511, 512) but autoimmunity of B cells has gained focus with identification of ectopic B cell follicles within MS CNS (499-502). These follicles can enable viral persistence and reactivation, but structural differences from lymphoid follicles suggest dysfunctional B cell maturation processes, supported by increased B cell activating factor (BAFF) (513). Lastly, Serafini *et al* (500) found fewer numbers of CTLs in SPMS, and more B cells.

#### *1.5.5.2. EBV viral load, DNA & RNA detection*

Other autoimmune diseases, like Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are correlated to increased EBV viral load compared to healthy controls (514, 515). However, for MS there have been conflicting results on measuring EBV viral load through DNA and RNA polymerase chain reaction (PCR) in peripheral blood, brain lesions and CSF. EBV has been detected in MS saliva (516), where it has been associated with increased disease activity. Significantly elevated EBV DNA has been identified in MS blood and CSF (329, 489), and in active relapse (517), but for the majority of cases, viral DNA levels do not associate with relapse/remission of MS (328, 518-520), or MS risk (479, 500, 521-530), including a meta-analysis undertaken by Santiago and colleagues (527). EBV (infected B cells, plasma cells or RNA) has been identified in post-mortem brains (324, 500, 513, 531-533) but this has failed to be replicated successfully (526, 534-540), while Sanders *et al.* (324) identified other herpesviruses in plaques. Pender *et al* (377) suggests the discrepancy in results is due to laboratory techniques, but overall it could mean that EBV dysregulation is a cause or effect of MS, and that viral load itself is not the primary cause of the disease, but instead reflects the humoral or cellular based pathogenesis.

#### *1.5.5.3. Host humoral response to EBV infection*

Antibodies both enable detection of immune responses against a pathogen and can functionally assist the immune system to detect and eliminate the pathogen. In MS, oligoclonal bands (OCB; IgG) are a hallmark of MS, and are present in approximately 90% of patients. Additionally, antibody-complement complexes have been found in CNS tissues of MS (541-543). Associations between EBV antibody levels and MS have been reviewed previously (384, 544). It has been consistently reported that later age of primary infection increases MS risk (4, 259, 453, 545, 546), and later age of primary infection can manifest as IM, which also increases MS

risk (166, 263, 452-454, 547-555). Serologically, MS cases are almost 100% seropositive compared to 90-95% of controls (449, 517, 556-559), which is a significant observation not seen for other herpesviruses (259, 341, 447, 517, 545, 546, 556, 560-563). Moreover, prospective studies show EBV infection as a prerequisite for MS (341, 545, 563-565), and being EBV seronegative significantly reduces MS risk (341, 449, 563). A range of EBV proteins have been measured in MS studies. Anti-VCA antibodies have shown 100% seropositivity in MS (518, 563, 566) with evidence for elevated levels in MS cases compared to healthy controls (545, 556, 557, 566, 567). Additionally high titres were associated with females, HLA "DR2", smokers (568) and loss of brain volume (569). Recently, Ruprecht *et al* (570) found elevated IgG against another capsid protein VP26 (BFRF3). Elevated antibodies in MS target EBNA complex (545, 557, 566), EBNA-2 (545), EBNA-3A,B,C (570), LMP1 (570) and lytic protein BRRF2 (478). The most prominent target for IgG in MS is latent protein EBNA-1. Although anti-EBNA-1 antibodies are common in healthy individuals (571), elevated IgG against EBNA-1 have been shown in MS continuously (198, 341, 478, 489, 545, 566, 570, 572, 573) as well as already elevated years before MS onset (564, 574). Thus, anti-EBNA-1 IgG is the strongest humoral risk factor for MS (166, 198, 299, 341, 570, 574, 575), when compared to other EBV antigens (341, 479, 574). Clinically, anti-EBNA-1 IgG levels have been linked to new (Gd) lesions, EDSS (489, 576, 577) and lesion size (576), although there has been some difficulty in replicating these observations (517, 518, 578). Anti-EBNA-1 IgG has been associated with HLA-DRB1*1501, but also found to be independently associated with MS (269, 459, 460). A further breakdown of the EBNA-1 specific immune response has been investigated by Sundstrom *et al* (198) who looked at overlapping peptides within the protein for antibody reactivity. They found the most significant peptides reactive to antibody in MS patients were EBNA-1$_{(421-440)}$ and EBNA-1$_{(431-450)}$. Using EBNA-1$_{(385-420)}$ which had the strongest statistical association in logistic regression (OR 12) regardless of HLA-DRB1*1501 status, increased risk

was found in DRB1*1501 positive and "high" EBNA-$1_{(385-420)}$ antibody titres. This EBNA-$1_{(385-420)}$ reactivity has also been demonstrated in IM individuals (579), although in this context it is notable that antibody reactivity was transient and was not normally maintained over time. Other studies have also investigated immunogenicity of overlapping EBNA-1 fragments in MS cohorts (572, 580, 581), with mixed significance. It is important to identify the minimal epitope region to reach significance between cohorts for investigating potential cross-reactivity or ability for pathogenesis. A study by Mechelli et. al. (582) reported on MS-discordant identical twins and found significance of EBNA-$1_{(401-411)}$ which has yet to be tested in a larger population cohort. Investigation of inflammatory modification of EBV targets, such as citrullinated autoantibodies against EBV, show promise as diagnostic tools for rheumatoid arthritis (RA; 630) but has yet to be explored for MS.

A hallmark of MS is the presence of OCB in the CSF (478, 500, 583-585). In MS, these CSF antibodies have been reported to be reactive against EBNA-1 (476, 478, 572, 583, 584, 586, 587), BRRF2 (478), VCA (476, 557), and alpha-beta crystallin (αβC) (586) but not postulated autoantigens such as MBP, myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (508). It is argued anti-EBV IgG is only a fraction of the total intrathecally synthesized IgG, as other pathogen targets have been identified including measles, rubella, Varicella Zoster Virus (VZV), Herpes Simplex Virus (HSV) and HHV-6 (326, 342, 588-593), suggesting bystander reaction of a chronic inflammatory process. It is theorized that this may be due to clonally expanded EBV infected B/plasma cells in the CNS (353, 594). Because other viruses (like measles) are not present in the brain, it is possible that there is recruitment of virus-specific plasmablasts to the inflamed CNS (595). Jafari *et al.* (572) reported significantly elevated EBNA-$1_{(394-451)}$-specific IgG in MS CSF and serum compared to non-inflammatory neurological disease patients. The anti-EBNA-$1_{(394-451)}$ IgG measurements in these two

compartments were significantly correlated, and when normalized by total IgG (which was significantly lower in MS serum and higher in MS CSF), no significant difference was seen between cohorts. From this it was suggested that the EBNA-$1_{(394-451)}$ IgG response was not intrathecally synthesized, instead the antibodies "leaked" into the CSF via blood-brain barrier (BBB) dysfunction. This theory is supported by evidence that OCB did not associate with the number of infected B cells in MS brain (500).

### 1.5.6. Theories on mechanisms of MS pathogenesis

A recent review by Pender *et al.* (377) summarizes the current supporting evidence for different theories behind MS pathology mechanisms. These encompass i) cross-reactivity, ii) bystander damage, iii) mistaken self hypothesis and the iv) EBV positive auto-reactive B cell hypothesis. The cross-reactivity theory is based upon EBV-specific T cells being cross reactive with CNS proteins (480, 491, 492), but being based solely on T cells, doesn't explain the presence of EBV infected B cells in the brain (500, 513, 531, 532). The bystander damage theory postulates that the autoimmune CNS response is secondary to an EBV antigen-targeted response (500), such as Epstein-Barr Virus-encoded small RNA (EBER) released from EBV infected cells activate innate immunity (531). This is supported by the presence of EBV-infected B cells in MS brain (500) and CD8+ T cells found close to plasma cells (488, 500). Pender *et al.* argued that EBV should be eliminated by the immune response and that the bystander damage theory doesn't explain identified autoimmunity (377, 596-598). Similar to the cross-reactivity theory, the mistaken-self theory or "αβC hypothesis" is based upon molecular mimicry: EBV-specific CD4+ T cells mistaking human homologous proteins (αβC) as foreign, inducing oligodendrocyte attack and resulting in demyelination (599). In support of this, EBNA-$1_{(385-420)}$ shares homology with αβC (198), EBV infection induces αβC expression in B cells (600), and αβC is expressed by oligodendrocytes and myelin (356, 599, 601). However, Pender *et al* (377)

said that this could only explain initial T cell mediated inflammation, with other specific T cells and EBV-infected B cells contributing to the persistence of inflammation (602). This could additionally occur through targeting MBP, as cross-reactive T cells have been identified (480, 491, 492, 603, 604), as well as EBNA-1 sharing homology with many human proteins with "crucial pathway roles" (605). EBV can infect human cerebral microvascular endothelial cells (606), and T cells against αβC are seen in MS (607). The most recent theory, supported strongly by Pender *et al* (377), is that EBV infects autoreactive B cells, enabling evasion of immune recognition, elimination and apoptosis. These infected cells accumulate in target organs, secrete pathogenic autoantibodies and present co-stimulatory signals to autoreactive T cells (353), causing release of human proteins (potential new antigens) from attacked CNS, furthering inflammation (377). EBV infected cells can inhibit autoreactive T cell apoptosis (608-610) and elicit inflammatory responses, facilitating macrophage and lymphocyte infiltration (611). With reported 20% of human naïve B cells being autoreactive (612), the infection of that population, along with a genetically determined defect (such as HLA-DRB1*15) in eliminating EBV infected B cells by CTLs could be underlying causative factors of MS. Although this theory is not proven, supporting evidence includes EBV infected B cells in MS brains (500, 531, 532), EBV+ autoreactive B cells in IM (613), decreased CTL immunity against EBV in MS (482) and germinal centres in MS brains. These centres can reactivate B cells, produce autoantibodies, and provide an explanation for the efficacy of B-cell-specific treatment approaches such as the anti-CD20 monoclonal antibody Rituximab (390) and immunotherapy with EBV specific CTLs (496). Further research into the identification of EBV infected autoreactive B cells in MS is required to prove this hypothesis.

### 1.5.7. EBV targeted preventative and therapeutic strategies for MS

Therapies for MS and EBV have targeted different aspects of the disease. General immunosuppressants and anti-inflammatory drugs/medications were outlined in Chapter 1.3.4. In light of the EBV role in MS pathogenesis, potential therapies have included anti-viral drugs against herpes viruses (Alacyclovir/valacyclovir), unfortunately these can't target latently infected B cells or act upon antibody-producing plasma cells (614-616). However, Rituximab targets CD20+ B cells, including EBV infected B cells, and has clinically shown promise (106, 617). Some B cell and BAFF targeting therapies have shown promise for other autoimmune disorders including RA and SLE, but are not yet used in MS (618). A vaccine against the gp350 envelope protein of EBV has been developed, aiming to induce an immune response before EBV can establish a latent permanent infection. This vaccine could not prevent asymptomatic EBV infection (619), but has been shown to reduce the frequency of IM (620). EBV-specific T cells have been used successfully in treating EBV-associated lymphoproliferative disease (621-623), and recently a similar approach was taken using autologous CTLs for a SPMS patient, showing substantial clinical improvement (624). If this research could be replicated and extended, using EBNA-1 as a target for T cell immunotherapy may emerge as an interesting therapeutic strategy (605, 625), although Pender *et al* (377) noted that CTLs could aggravate CNS inflammation, and so cautioned against the use of EBNA-1 specific T cell intervention in RRMS.

## 1.6. Summary of Background and Development of Thesis Questions

MS is clearly a multifaceted disease, with several risk factors contributing to disease development. However, a conceptual understanding of the numerous influencing factors (DRB group alleles rather than just dichotomy of HLA-DRB1*1501 presence; specific EBV epitope targets rather than just history of IM) is essential to better classify and stratify risk of MS, at both a familial and population levels. Specific questions related to the role of EBV in the pathogenesis of MS were investigated using a Western Australian (WA) cohort in this thesis:

(i) Can anti-EBNA-1 and anti-VCA IgG antibodies discriminate MS patients from healthy controls in the WA cohort?

(ii) Is it possible to create an immunoassay to measure antibody reactivity against the B cell epitope EBNA-$1_{(398\text{-}413)}$, and does this aid in discriminating MS cases from healthy controls?

(iii) Does post-translational modification (citrullination) of EBNA-$1_{(398\text{-}413)}$ influence its antigenicity, compared to non-modified EBNA-$1_{(398\text{-}413)}$, and does this contribute to risk analysis?

(iv) Using the EBNA-$1_{(398\text{-}413)}$ assay, which of the IgG subclasses is most prevalent and is there a difference between MS cases and controls?

(v) Are MS serum antibodies and EBNA-$1_{(398\text{-}413)}$ specific IgG antibodies cross-reactive to an array of brain proteins and how does this compare to healthy controls?

(vi) Does MS serum containing these potentially cross-reactive antibodies have a detrimental viability effect on brain cells *in vitro*?

# 2. Materials & Methods

## 2.1. Universal Precautions

To prevent any contamination between samples as well as samples to researcher, all samples obtained from human individuals were treated as if containing infectious agents. Each method was conducted following universal biosafety measures and including personal protective equipment (PPE) and effective hand-washing/disinfection procedures when leaving the laboratory. Murdoch University and the Institute for Immunology and Infectious Diseases (IIID) Laboratory Safety and Precautions were undertaken at all times. Preparation of PCR master mixes was conducted in a specially designated class II biosafety hood with laminar flow, and subsequent pre- and post-PCR experiments were performed in separate self-contained rooms. Cell lines were kept in a Physical Containment Level 2 (PC2) classification room until proved non-infected. Laboratory work areas and materials including benches, hoods and pipettes were decontaminated prior and after use with 70% ethanol or the detergent Viraclean (Statewide Cleaning Supplies, Australia) according to the accredited laboratory safety manual.

## 2.2. Patient samples

A total of 426 MS patients in the Perth (Western Australia) Demyelinating Disease Database (PDDD) from the cohort previously described (184) were included in the study. The control cohort (n=186) was established from the population of Busselton, Western Australia (626). Serum samples from both cohorts were collected and stored at -80°C until tested. Within the final year, patients who returned to clinic from the PDDD cohort had additional serum samples taken, and of these a subset had peripheral blood mononuclear cells (PBMCs) collected, additional to their clinical history. These samples were collectively named ‘PDDD-b’ (n=32), from which eight samples were selected for experiments, as summarized in Table 2-1. “Acute patients” refers to MS patients where samples were taken during a relapse of symptoms.

Healthy control samples were also collected from Red Cross buffy coat donations (n=34), and ten samples were selected based on HLA-DRB alleles.

**Table 2-1. Summary of PDDD-b Multiple Sclerosis (MS) and Red Cross (RC) samples.**

| Sample | Base ID | Gender | HLA-DRB1.1 | HLA-DRB1.2 | MS/RC | Clinical Information |
|---|---|---|---|---|---|---|
| **1** | 65499 | Male | 03:01 | 15:01 | MS | Acute relapse |
| **2** | 65556 | Female | 15:01 | 15:01 | MS | CIS |
| **3** | 65514 | Male | 03:01 | 15:01 | MS | Acute relapse |
| **4** | 65508 | Female | 03:01 | 03:05 | MS | SPMS, not on treatment |
| **5** | 65672 | Female | 11:01 | 13:01 | MS | SPMS, fingolimod |
| **6** | 65720 | Female | 04:04 | 13:02 | MS | SPMS, not on treatment |
| **7** | 65726 | Female | 07:01 | 15:01 | MS | SPMS, interferon |
| **8** | 65715 | Male | 03:01 | 15:01 | MS | PPMS, not on treatment |
| **9** | 52477 | Female | 10:01 | 15:01 | RC | |
| **10** | 63665 | Female | 03:01 | 15:01 | RC | |
| **11** | 63647 | Female | 04:05 | 15:02 | RC | |
| **12** | 66382 | Female | 04:04 | 08:01 | RC | |
| **13** | 63658 | Male | 01:03 | 04:04 | RC | |
| **14** | 64308 | Male | 03:01 | 04:01 | RC | |
| **15** | 64488 | Female | 04:02 | 12:01 | RC | |
| **16** | 57300 | Female | 04:04 | 07:01 | RC | |
| **17** | 61254 | Male | 14:01 | 14:01 | RC | |
| **18** | 64467 | Female | 03:01 | 13:01 | RC | |

PDDD: Perth Demyelinating Disease Database. ID: Identifier. HLA: Human Leukocyte Antigen. CIS: clinically isolated symptom/s; first presentation of MS. SPMS: Secondary Progressive MS. PPMS: Primary Progressive MS.

## 2.3. Materials and Media

For catalogue numbers of kits and reagents purchased, see Supplementary Table S-1.

### 2.3.1. Enzyme Linked Immunosorbent Assay (ELISA) reagents

#### *2.3.1.1. Commercial ELISA kits*

Two different ELISA kits were purchased (DiaSorin, Australia) for detection of IgG against EBNA-1 and VCA. The sample diluent provided with the kits was used to dilute samples tested in commercial and subsequent in-house ELISAs.

#### *2.3.1.2. ELISA plates*

Streptavidin-coated 96 well plates (ThermoFisher Scientific, Australia) were used for all in-house ELISAs.

#### *2.3.1.3. In-house ELISA peptides*

A consensus sequence of EBNA-1 derived from individual MS patient sequences (instead of using the sequence of the laboratory strain of EBV B-98-5) was used to produce the B cell epitope: Epstein-Barr Virus Nuclear Antigen-1 ($\text{EBNA-1}_{(398\text{-}413)}$) PPPGRRPFFHPVGEAD (sequence selection method described in Chapter 2.4.7; amino acid sequence table in Supplementary Table S-2). This peptide was synthesized to 95% purity with reversed-phase high-performance liquid chromatography (RP-HPLC; Mimotopes Australia) with an SGSG sequence spacer at the N-terminus which was biotinylated to enable binding in the correct orientation to the streptavidin coated plates. The peptide sequence of the synthesized product was independently confirmed (Proteomics Node, Perth, Australia). The peptide was re-constituted with dimethyl sulfoxide (DMSO) to 10 mg/ mL, aliquoted and stored at -20°C until needed. A second peptide was synthesized to identify IgG antibodies against a citrullinated

form of the B cell epitope. The EBNA-$1_{(398-413)}$ sequence was modified to contain two citrullinated arginine residues at aa 402-403. The peptide was reconstituted and stored identically to the first peptide, with the amino acid sequence confirmed by Proteomics.

#### *2.3.1.4. Phosphate Buffer Solution (PBS)*

A concentrated 10X PBS was made by dissolving 25 tablets (Sigma Aldrich, Australia) into 500 mL autoclaved water. The buffer was subsequently filter sterilized using a Steritop filter unit (Merck Millipore, Australia) and stored at 4°C. Buffers requiring 1X PBS were made from diluting stock buffer 1:10.

#### *2.3.1.5. In-house PBS-Tween (PBST) wash buffer*

In-house wash PBST buffer was prepared by adding 0.01% Tween-20 (BDH, Australia) to 1X PBS buffer.

#### *2.3.1.6. In-house Coating Buffer*

Coating buffer was prepared using 1X PBS with the addition of 0.1% Bovine Serum Albumin (BSA; Sigma Aldrich, Australia) and 0.1% Sodium Azide (Sigma Aldrich, Australia) and used for all in-house ELISAs.

#### *2.3.1.7. In-house Secondary Antibody Diluent*

Secondary antibody diluent was made using 1X PBS supplemented with 2% BSA and was stored at 4°C, for up to a week.

#### *2.3.1.8. In-house ELISA IgG detection antibodies*

Separate secondary antibodies were purchased to detect total IgG (Sigma Aldrich, Australia) and three IgG subclasses; IgG1, IgG2 and IgG3 (Sapphire Biosciences, Australia).

#### *2.3.1.9. Tetramethylbenzidine (TMB)*

3, 3', 5, 5' Tetramethylbenzidine (TMB) Liquid Substrate (supersensitive) was purchased (Sigma Aldrich, Australia) and stored at 4°C, and used as a colourmetric substrate for in-house ELISAs.

#### *2.3.1.10. In-house Stop Solution 1 N $H_2SO_4$*

1N Sulphuric acid ($H_2SO_4$) solution was prepared using deionized water in a sterile hooded cabinet, and stored in a dark hazardous chemicals cabinet.

### **2.3.2. Antibody Isolation using affinity columns**

#### *2.3.2.1. Tris-Buffered Saline (TBS)*

TBS was used for antibody capture using affinity columns (ThermoFisher Scientific, Australia) and prepared as per manufacturer instructions, with a final concentration of 25 mM Tris, 0.15 M NaCl, pH 7.2.

#### *2.3.2.2. 5M NaCl stock solution*

5M NaCl was prepared in deionized water and autoclaved, then kept at room temperature.

#### *2.3.2.3. Column wash buffer*

5 M NaCl added to "acetate wash buffer" (ThermoScientific, Australia) so that final concentration was 0.012 M NaCl.

#### *2.3.2.4. Neutralization buffer*

1 M Tris (Sigma Aldrich, Australia) in ultrapure water and kept at 4°C.

#### *2.3.2.5. Column cleaning buffer*

Cleaning buffer was prepared by 1X TBS with 1M NaCl (from 5M NaCl stock). Stored at 4°C.

### 2.3.3. Protein macroarray

#### *2.3.3.1. Macroarray*

A HexSelect Macroarray was purchased (SourceBioscience, Germany) and kept at room temperature until needed.

#### *2.3.3.2. 3 M HCl*

3 M HCl was prepared and stored in a hazardous cabinet.

#### *2.3.3.3. Tris-HCl, pH 7.5*

1M Tris was made in deionized water, pH adjusted to 7.5 using 3M HCl. The bottle was autoclaved and stored at room temperature.

#### *2.3.3.4. In-house TBS Buffer*

Stock 1X TBS Buffer was made as 10mM Tris-HCl (pH7.5) with 500mM NaCl. The solution was autoclaved and stored at room temperature.

#### *2.3.3.5. TBS-Tween 20 (TBST) Buffer*

Final buffer was made to 20mM Tris-HCl (pH 7.5), 500mM NaCl with 0.05% Tween-20.

#### *2.3.3.6. TBS-T-Triton X100 (TBST-T) Buffer*

Final buffer was made to 20mM Tris-HCl (pH 7.5), 500mM NaCl with 0.05% Tween-20 and 0.5% Triton-X 100 (Sigma Aldrich, Australia).

#### *2.3.3.7. Blocking Buffer*

TBS-T buffer was prepared with 3% skim milk powder (HomeBrand, Australia).

#### *2.3.3.8. Enhanced Chemiluminescence (ECL) Substrate*

ECL substrate (BioRad, Australia) was made at a 1:1 ratio with the Peroxidase substrate and Luminos substrate immediately before addition to the protein array.

#### *2.3.3.9. Macroarray Cleaning Buffer*

TBS-T buffer was prepared with 5% skim milk powder.

#### *2.3.3.10. 1M Tris-HCl, pH 6.8*

1 M Tris buffer was made, adjusted to pH 6.8 using 3 M HCl. Bottle was autoclaved and stored at room temperature.

#### *2.3.3.11. Macroarray stripping Buffer*

As instructed by the manufacturer, 2% sodium dodecyl sulphate (SDS; Astral Scientific Pty Ltd, Australia) was prepared using 65.5 mM Tris-HCl (pH 6.8) with 100 mM beta-mercaptoethanol (Sigma, Australia). The buffer was heated in a water bath to 70°C before use.

#### *2.3.3.12. Dot Blot Blocking Media*

1 X PBS was supplemented with 5% BSA (Sigma Aldrich, Australia).

### 2.3.4. Cell culture

#### *2.3.4.1. Foetal Bovine Serum (FBS)*

FBS (Scientific Partners, Australia) was heat inactivated (56°C) for 30 minutes and aliquoted in 50 mL tubes and frozen at -20°C until needed.

#### *2.3.4.2. Neuroblastoma SH-SY5Y cell line*

SH-SY5Y is a human-derived cell line isolated from bone marrow of a four year old female with neuroblastoma. The cell line was a kind gift from Prof. Ian Mullaney's group at Murdoch University. The cell line was received at passage 24 and grown in SH-SY5Y media.

#### *2.3.4.3. SH-SY5Y media*

1:1 volume of DMEM High glucose (Sigma Aldrich, Australia) and Ham's Nutrient F12 media (Sigma Aldrich, Australia) + 5% FBS (Serana, Australia), supplemented with 1% Penicillin-Streptomycin-Glutamine (Invitrogen, Australia). Media was kept refrigerated at 4°C, and warmed to 37°C before use with cells.

#### *2.3.4.4. Oligodendrocyte MO3.13 cell line*

MO3.13 is a hybrid cell line from a mutant of human rhabdomyosarcoma with adult human oligodendrocytes, exhibiting markers of immature oligodendrocytes. Differentiated MO3.13 have been shown to express MBP and MOG. This cell line was a kind gift from Prof. Guillemin's group from Macquarie University with the blessing of Prof. Cashman from University Toronto. The cell line was received at passage 6 and grown in MO3.13 media as provided by Prof. Guillemin's group.

#### *2.3.4.5. MO3.13 media*

RPMI 1640 media (Gibco, Australia) was supplemented with 10% FBS and 1% Antibiotic-Antimycotic (Gibco, Australia). The media was kept refrigerated at 4°C, and warmed to 37°C before use with cells.

## 2.4. Methods

### 2.4.1. Tracking of samples

To ensure traceability for the large number of samples handled in this thesis, all reagents and samples were labelled and recorded with unique identifiers known as “Base IDs”. This process de-identifies personal information on patients including their name, but can be linked to the Universal Medical Record Number (UMRN), which is given to a single individual and can be used to determine if samples are from different time points of a single individual. This Base ID is linked to the type of sample and storage location on an in-house database system (EpiLab) which allows tracking of samples. To minimize risk of contamination or mistake between samples, a second person checked, prior to an experiment, the racking order and Base IDs of samples as well as locations of positive and negative controls. All reagent batch/lot numbers were also recorded for experiments. This can help trouble shooting in case an experiment failed.

### 2.4.2. Blood separation and PBMC storage

Whole blood samples from PDDD-b were collected into commercial tubes for blood separation; Beckton Dickinson (BD) Ethylenediaminetetraacetic acid (EDTA; BD, Australia) for plasma and buffy coat and serum-separating tubes (SST; BD, Australia) for serum. After registration of samples using IIID’s non-identifying base ID, tubes were centrifuged (1300 x *g*, 10 minutes) using Beckman Coulter Allegra X-15R. Serum was aliquoted from the SST tube, plasma and buffy coats were aliquoted from the EDTA tube and stored at -80°C. Frozen buffy coats were later used for DNA extraction with subsequent HLA typing.

For Red Cross buffy coat bags, 7 mL was also allocated into a 15 mL falcon tube undiluted. The remaining sample was then diluted 1/3 RPMI and 30 mL of this layered over 15 mL Ficoll

(GE Healthcare, Australia) in 50 mL falcon tubes. All tubes were centrifuged 800 x *g* for 15 minutes with the brake off. After centrifugation, the 15 mL tube was used for plasma and buffy coat collection, which was aliquoted and stored at -80°C.

### 2.4.3. DNA extraction from buffy coat samples

The PDDD MS cohort had DNA extracted using multiple commercial DNA extraction kits (Qiagen, Genfind & Promega, Australia). For samples from the Red Cross and newly recruited MS patients in PDDD-b, DNA extraction used commercial kits (Qiagen, Australia) as per manufacturer's instructions and DNA was stored at 4°C.

### 2.4.4. HLA typing

Sequence based HLA typing of both PDDD (MS) and control cohort had been performed prior to project commencement as previously described (169, 184). HLA typing was additionally done for individuals within cohort PDDD-b but not PDDD. For both PDDD and PDDD-b, genotyping was performed using PCR with HLA locus specific primers followed by subsequent sequencing on the ABI Prism 3730 and 3730xl Genetic Analysers. Sequence editing was carried out using ASSIGN V4.0.1.36 (Conexio Genomics). All HLA typing results for MS and healthy control samples were resolved to at least the 4-digit level using heterozygous ambiguity resolving primers where applicable. HLA typing was carried out by the accredited staff at IIID.

### 2.4.5. Automation (and documentation) of sample preparation and ELISA protocol

Samples were serially diluted using a BioMek FX$^P$ laboratory automation station (Beckman Coulter), and ELISA protocols were set up on a BioMek FX (Beckman Coulter) and an ELx 405 washer (BioTek, Vermont, USA) to minimize pipetting error. Both automation processes required the utilization of the BioMek FX software, with user-configured experimental methods to optimize deck layout, required reagents, lab-ware, and liquid-handling techniques, pipetting and associated parameters including sample and reagent volumes, aspiration and dispensing speeds, heights, mixing and tip touching (adapted from (626)).

### 2.4.6. Serum sample preparation

MS cohort, healthy controls and internal assay control serum samples were serially diluted using a BioMek FX$^P$ robot to 1:1075, using sample diluent provided by the commercial EBNA-1 ELISA kit (DiaSorin), and refrigerated overnight. The following day, samples were thoroughly mixed on an orbital shaker before both commercial and in-house ELISAs were performed on the same day from the same sample dilutions.

### 2.4.7. EBNA-1 B cell epitope sequencing

EBNA-1 sequencing was carried out by Dr. Monika Tschochner prior to commencement of this project. Briefly, DNA of MS samples from PDDD was isolated from buffy coats with an automated robotic setup using DNA isolation kit (Genfind, Australia). A minority of samples was manually extracted using QIAamp Mini extraction kits (Qiagen, Australia) following the manufacturer's instructions. A novel semi-nested PCR approach with a fully automated setup utilising Biomek FX robots was employed. All primers used for amplification have been

previously published (627-629) and have been named according to the position in the reference strain B95-8. First round PCR was performed using High Fidelity Taq (Roche, Australia) in a 25µl reaction with primers EBV109111F-EBV109951R (at 25pmol/µl), resulting in an 840 base pair (bp) fragment. A semi-nested PCR was followed using the primer combination EBV109111F-EBV109869R resulting in a final 749bp product. Alternatively, a shorter semi-nested PCR was performed using the primer combinations EBV109111F-EBV109759R (648 bp) and EBV109111F-109459R (348bp) respectively. Successful PCR products were purified with AMPure (Beckman Coulter, Australia), using an automated setup and sequenced with the PCR primers at a concentration of 1 pmol/ul. Sequencing products were purified with magnetic beads using the Cleanseq (Beckman Coulter, Australia) protocol according to the company's instructions at IIID's protocol, followed by sequencing on an automated 96 capillary ABI 373 DNA sequencer. Sequences were edited with the ASSIGN V1.0.2.45 software (Connexio) and fasta files generated and aligned using Bioedit for subsequent analysis.

#### 2.4.8. In-house EBNA-1$_{(398-413)}$ ELISA

As shown in Figure 2-1, Streptavidin-coated plates were washed 3x with 300µl PBST using an ELx405 Microplate Washer (BioTek, Vermont, USA) and incubated at room temperature for 1 hour with 100µl EBNA-1$_{(398-413)}$ or EBNA-1$_{(398-413\ CIT)}$ biotinylated peptides (0.5µg/ mL) in in-house coating buffer. Plates were washed (4x 300µl PBST), and incubated for 1 hour at room temperature on a PHMP plate shaker (Thermo Fisher Scientific) after serially diluted internal assay controls and either MS or healthy cohort samples were added in separate wells. Plates were washed (4x 300µl PBST) and incubated with 100µl anti-human IgG (Fc specific)-Peroxidase antibody for 1 hour at room temperature. Following incubation, plates were washed again (4x 300µl PBST, 2x 300µl PBS only) and incubated with 100µl super-sensitive TMB liquid substrate for 30 minutes before stopping the reaction with 100µl $H_2SO_4$. Data was

recorded as optical density (OD) at 450nm on a DTX 880 Multimode detector spectrophotometer (Beckman Coulter), and lower cut-off was set at 3 standard deviations above the mean $OD_{450nm}$ of the negative control for all assays ($OD_{cutoff}$ = 0.039).

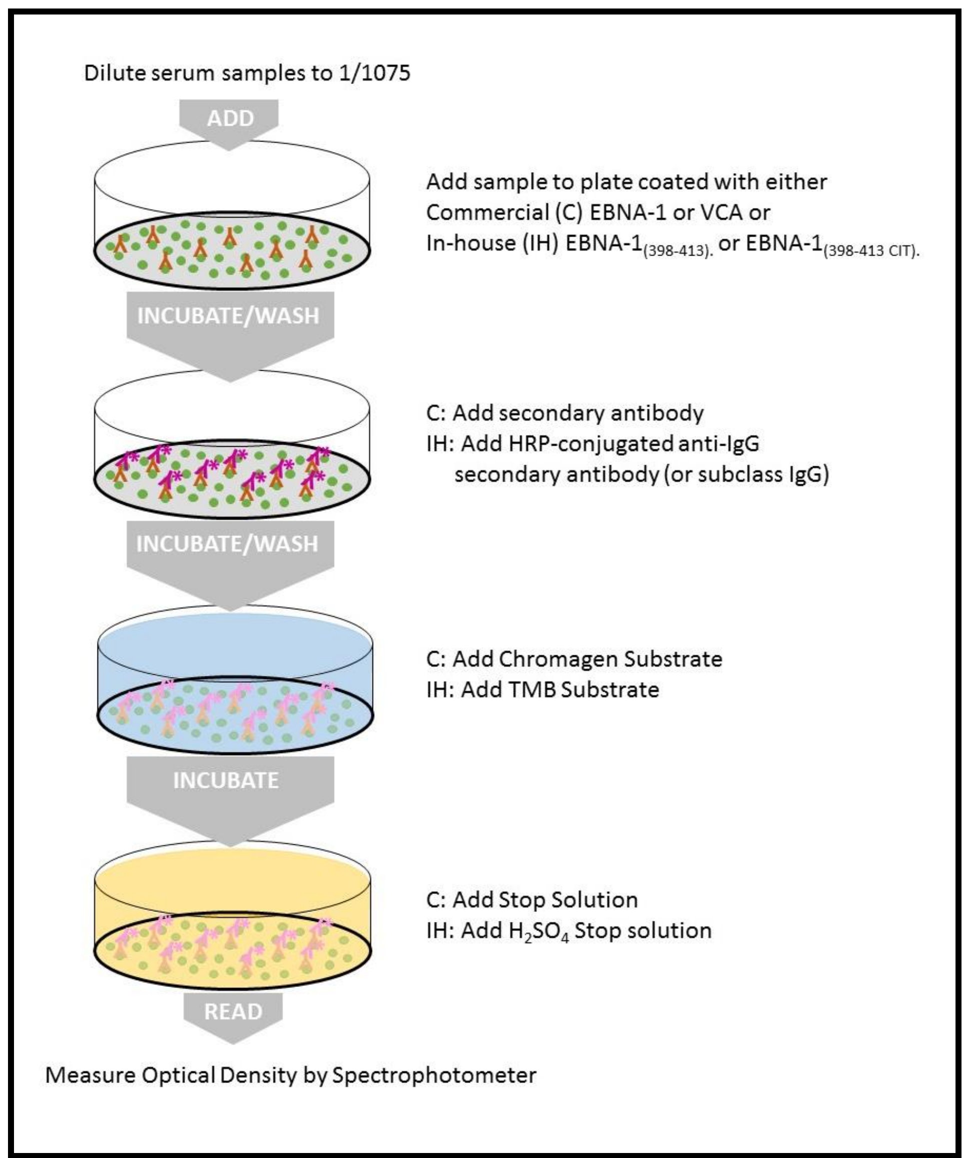


**Figure 2-1. Overview of the in-house ELISA method**. Each of the steps illustrated was automated using the Biomek FX robot. C: Commercial. IH: In-house.

#### 2.4.9. Commercial EBNA-1 and VCA ELISA

Commercial ELISAs (DiaSorin, Australia) were used to determine IgG antibody levels against EBNA-1 and VCA proteins. The assays were carried out according to the manufacturer's instructions with the exception that all samples were serially diluted to 1:1075 to be able to measure samples within the linear range of the assay (see Chapter 3). Data was recorded as optical density (OD) at 450nm-620nm, as described for the in-house ELISA protocol, and lower cut-off was set at three standard deviations above the mean $OD_{450\text{-}620nm}$ of the negative control across all plates for EBNA-1 ($OD_{cutoff} = 0.031$) and VCA ELISA ($OD_{cutoff} = 0.057$). In-house internal triplicate controls were also included, as discussed in Chapter 3.

#### 2.4.10. In-house EBNA-1$_{(398\text{-}413)}$ IgG subclasses ELISA

This assay utilized the method described in 2.4.8, but following washing after sera sample incubation, plates were incubated with 100µl anti-human IgG1, IgG2 or IgG3-specific HRP-conjugated secondary antibody for 1 hour at room temperature. Data were recorded as optical density (OD), as described previously and lower cut-off was also set at 3 standard deviations above the mean $OD_{450nm}$ of the negative control for all assays (IgG1 $OD_{cutoff} = 0.041$; IgG2 $OD_{cutoff} = 0.062$; IgG3 $OD_{cutoff} = 0.043$). Any plates for which triplicate or serial dilution controls were not within the accepted variance range were re-tested in full.

#### 2.4.11. IgG subclass antibody capture and purification

Serum samples were chosen based upon clinical information and in-house ELISA antibody titres, to use with a modified spin column-based Biotinylated Protein Interaction Pull-Down Kit (Thermo Fisher, Australia) to collect purified anti-EBNA-1$_{(398\text{-}413)}$ specific antibodies. The columns were used according to manufacturer's instructions, all at room temperature, and

centrifuged at 1250 x *g* for 60 seconds as mentioned. Briefly, 50µL immobilized streptavidin (STP) "slurry" was pipetted into a spin column and washed 5 times with TBS, centrifuging to remove the buffer. Next, 50µg EBNA-$1_{(398-413)}$ peptide in PBS was incubated with STP beads for 1 hour on a rocking platform ("rocker", Ratek) at medium speed. The columns were centrifuged and 250µL Biotin Blocking Solution was added and incubated for 5 min before centrifugation three times, then washed 3 times with TBS. Following optimization to ensure the column was not overloaded, sera samples were diluted 1:2 and incubated with the EBNA-$1_{(398-413)}$ peptide-bound beads for 1 hour on a rocker. The bead suspension was centrifuged and washed 4 times with column wash buffer, followed by the addition of 250µL elution buffer, which was incubated for 5 min and final centrifugation into a collection tube with 10µL Neutralization buffer. This elution procedure was repeated once to obtain sufficient antibody for subsequent experiment. Eluted antibodies were initially kept at 4°C. An aliquot of eluted antibodies was tested using EBNA-$1_{(398-413)}$ in-house ELISA to confirm presence of total and subclass IgG antibodies before use with the protein macroarray or freeze drying for subsequent experiments.

#### 2.4.12. Protein macroarray

HexSelect High density protein macroarrays were purchased through Source BioScience (Germany). The method followed the manufacturer's instructions, performing all steps at room temperature, with gloves and tweezers so as to not touch the blotted proteins on the membrane. Briefly, the array was rinsed with 96% ethanol and then ultra-pure $H_2O$ to remove traces of ethanol. In a new plastic tray, the array was washed 2 x 10min in TBST-T while on a rocker for gentle agitation. The array was rinsed twice in TBS, and then washed 2 x 10min in TBS on a rocker. The array was then incubated for 2 hours in blocking solution on the rocker (20 mL in sealed bag) after which primary isolated antibody (200ul each) was added to the blocking

solution so that each antibody was at a 1/100 dilution. The array was incubated on the rocker for 16 hours. The following day, the array was washed 3 x 10min in TBS-T, and incubated for 2 hours in 20 mL secondary antibody (anti-human IgG, Sigma Aldrich) in 20 mL blocking buffer in a sealed bag on a rocker. After incubation, the array was washed 4 x 10min in TBS-T, 2 x 10min in TBS and finally transferred to a new tray and incubated for 5 min with made ECL substrate. The array was placed between two plastic sheets and images were taken using the Fusion FX (Fisher Biotec, Australia) using single imaging for blot and accumulative images for ECL ("chemiluminescent" and "blot marker"). For the first array, the same blot was used for re-incubation of primary sample and blocking buffer after 3 x 10 min washes with cleaning buffer, before imaging. To re-use the same array for a second experiment using different samples, stripping the array was carried out by washing the array with heated stripping buffer for 30 minutes, before drying the array between blotting sheets until further use.

#### 2.4.13. Protein macroarray positive control dot blot

To ensure optimal performance of reagents, a positive control dot blot was performed in parallel to each macroarray using the following method, with blocking and washing occurring on a PHMP shaker (Thermo Scientific, Australia). Nitrocellulose membrane (GE Healthcare, Germany) was blotted with EBNA-$1_{(398-413)}$ peptide at 1ug/ mL concentration and left to dry. The membrane was washed 3 x 5 min in PBST, then submerged in blocking media for 30 min on a shaker. After further washing steps (3 x 5 min) in PBST, pooled positive control MS serum was added in serial 2-fold dilution to the membrane (from 1:2 to 1:32). After 30 min room temperature incubation, the membrane was washed again 3 x 5 min PBST. Next the membrane was incubated with ELISA secondary anti-IgG HRP-conjugated antibody for 1 hour on a rocker at medium speed, followed by 3 x 5 min washing in PBST and 1 x 5min in PBS. Finally approximately 1 mL of Clarity ECL was added and incubated for 5 min before excess ECL was

removed and the blot was taken to the imager for immediate analysis. An example of the dot blot controls is seen in Supplementary Figure S-1.

### 2.4.14. Analysis of hexSelect macroarrays

The array was 30cm x 30cm in size, with proteins blotted in duplicate onto the membrane in a 5 x 5 square surrounded a black ink dot. The protein array blotted proteins therefore have a location between X1 to X240 and Y1 to Y240. Read-out of the macroarray was time sensitive for signal detection, and so control blots and macroarray were imaged immediately following the ECL addition stage. The Fusion camera stage and height of camera did not allow a high resolution photo to be taken of the entire array in one image. Therefore, an overlaying plastic sheet with a 3 x 3 square grid was placed on top of the array, and images were taken of each grid square (1 to 9) with white light (Figure 2-2A) and Chemiluminescent settings (Figure 2-2B). This allowed the highest resolution of image while maintaining the least number of photos taken (due to time sensitivity). Fusion software saved the captured images as Tagged Image File Format (TIFF). These were converted to Bitmap Image File (BMP), and chemiluminescent images resolution changed to 2048 pixels x 2048 pixels to match the size of the white light images of the macroarray. These images were layered using Pixlr Editor to identify location of ECL-positive dots which appear as black on a grey background. A layering tool enabled a red square to identify a 5 x 5 blotted square around a black ink dot to match the chemiluminescent signal with X/Y location on the array, seen by the white light image. These signal locations were mapped by their X/Y axis location, as shown in Figure 2-2D&E where they were checked for duplicate matches by codes provided by the manufacturer. Only duplicate matches were accepted as true results.

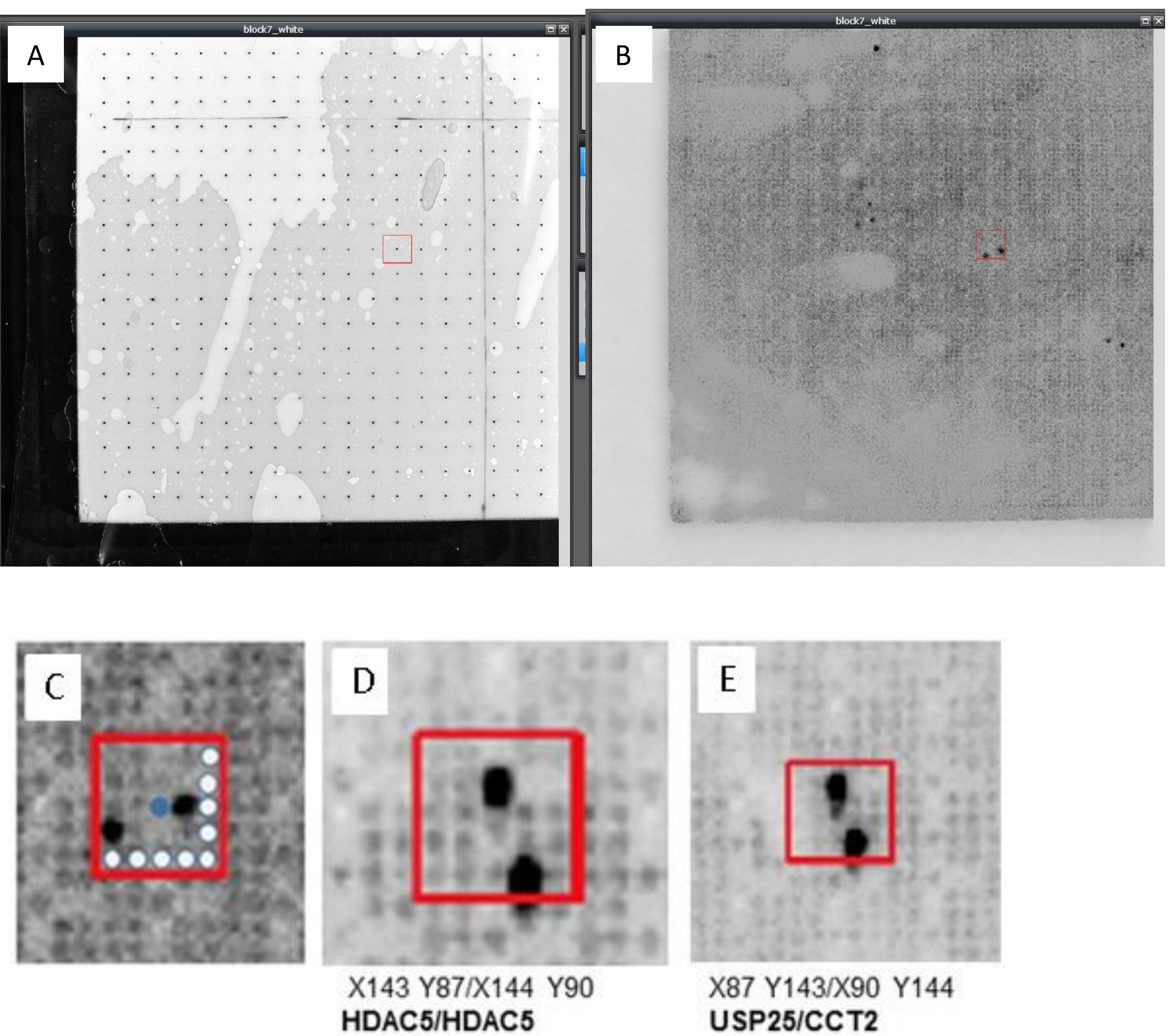


**Figure 2-2. Analysis of protein macroarray. (A)** White light "dot blot" image in Pixlr software. (B) Chemiluminescent image in Pixlr software of equal resolution for comparison, with red square on additional layer. (C) Example of 5 x 5 square of dotted proteins (ink dot as a blue circle), with signal higher than background shown as two black dots. (D) X/Y locations of two dots identify matching protein codes from the manufacturer. (E) Two signals identified, but with mismatched protein codes, and thus excluded reactivity.

### 2.4.15. Cell culture

Cells were taken from an existing line (P6 for MO3.13 and P24 for SH5Y) and grown in media as (3.2.22 and 3.2.24 respectively) at 37°C with 5% $CO_2$ (Sanyo Incubator, Australia). Media was changed every 3-5 days, and once confluence reached about 70%, cells were washed with PBS, then resuspended using 2ml of trypsin for no longer than 2 min (Trypsin-EDTA, Sigma

Aldrich) and washed with fresh media before splitting 1/5 or 1/10 into a new flask with fresh media. Viability and cell counts were maintained to ensure consistent growth, morphology and preparation for experiments. Photographs of cell cultures demonstrating cell morphology and count were taken using a Nikon Eclipse TS100 microscope with 40X magnification.

#### 2.4.16. Cell counts

Cells were counted manually with a Nikon Eclipse TS100 microscope using trypan blue exclusion. After trypsinisation and washing of cells with media, 10ul cell media suspension was added to 10ul 0.4% trypan blue (Life Technologies, Australia) and mixed thoroughly before adding onto a Blaubrand haemocytometer (Sigma Aldrich, Australia) to calculate cell concentration. Trypan blue is a dye which can enter cells with disrupted cell membrane integrity. Dead or nonviable cells are stained by the dye, appearing dark blue and are excluded for the live cell count. Cells were counted in four squares of the haemocytometer according to the manufacturer's instructions. Average cell count per square was calculated and multiplied by the dilution factors used to report cell counts per millilitre.

#### 2.4.17. Testing cell lines for *Mycoplasma* contamination

Following an in-house protocol, both cell lines were tested for *Mycoplasma* contamination before use. Briefly, 2 x $10^6$ cells were extracted after growth in antibiotic free media for two days. DNA was extracted using QIAamp (Qiagen, Australia) DNA extraction method followed by PCR using specific primers (Table 2-2) for amplification of *Mycoplasma* DNA (*M. argini, M. hominis, M. hyorhinis, M. fermentans* and *M. pharynges*) if present. Cell lines must be tested before use to ensure results are not influenced by metabolic products of *Mycoplasma*. Extracted DNA was diluted to 40 ng/ul for *Mycoplasma* PCR, and added to PCR Master Mixes

(Invitrogen) including the forward and reverse primers for Mycoplasma testing (LeedmanMyco) and positive internal controls (Human Growth Hormone; HGH) (Table 2-3). These underwent PCR (Biorad C1000 Thermal Cycler, Australia) with conditions as outlined in Table 2-4. Samples and controls were tested in duplicated, and products confirmed as *Mycoplasma* contaminated or uncontaminated by gel electrophoresis.

**Table 2-2. Primer sequences for PCR reactions.**

| Primer Name | Primer Sequence |
|---|---|
| **LeedmanMycoF (forward)** | 5`- GGG AGC AAA CAG GAT TAG ATA CCC T-3` |
| **LeedmanMycoR (reverse)** | 5`- TGC ACC ATC TGT CTC TCT GTT AAC CTC- 3’ |
| **HGH (forward)** | 5`-TAT CCC AAA GGA ACA GAA GTA TTC ATT-3` |
| **HGH (reverse)** | 5`-TGT TTG TGT TTC CTC CCT GTT GGA-3` |

**Table 2-3. Reagent concentrations for PCR reactions**

| Reagent | Initial concentration | Final concentration | x1 rxn volumes (µL) |
|---|---|---|---|
| **PCR buffer** | 10x | 1x | 2 |
| **$MgCl_2$** | 50 mM | 3 mM | 1.2 |
| **dNTP** | 10 mM each | 0.25 mM | 0.5 |
| **Forward primer** | 10 µM | 0.25 mM | 0.5 |
| **Reverse primer** | 10 µM | 0.25 mM | 0.5 |
| **Platnium Taq** | 5 Units/µL | 0.025 Units | 0.1 |
| **Water** | | | 13.2 |
| **DNA** | 40 ng/µL | 80 ng | 2 |

Rxn: reaction

**Table 2-4. PCR temperature cycle**

| Cycle | Temperature | Time |
|---|---|---|
| **Pre-cycle** | 95°C | 5 minutes |
| **Cycle (40x)** | 95°C | 15 seconds |
| | 55°C | 15 seconds |
| | 72°C | 15 seconds |
| **Post cycle** | 15°C | ∞ |

#### 2.4.18. Effect of MS serum on cell cultures

Cells were cultured in 24 well Nunc plates (Thermo Scientific, Australia) with MO3.13 or SH-SY5Y cell media, with or without acute MS pooled serum (n=3 from Table 2-1[1-3]) in triplicate. Plates were incubated at 37°C, 5% $CO_2$. One of the identically cultured wells was analysed at 6, 24 and 48 hours. Cells were washed with PBS, trypsinized and counted. Manual viability was determined using the method from 2.4.16.

## 2.5. Statistical analysis

Comparisons between cases and controls of HLA risk groups and positivity of samples from the commercial and in-house ELISA were carried out using Pearson's Chi-squared tests or Fisher exact tests as appropriate. ELISA OD values were analysed on the log (base 10) scale to normalize data, with analyses based on multiple linear regression models/ANOVA, with dummy group covariates as applicable. OD values exceeding the upper limit of measurement (OD 4.0) were incorporated via normal-based censored linear regression. Case-control logistic regression was used to assess the joint influence of VCA, EBNA-1, EBNA-$1_{(398\text{-}413)}$ IgG and subclasses and EBNA-$1_{(398\text{-}413\ CIT)}$ antibody levels, as well as gender, age and HLA-DR results on MS disease risk. Cell viability was also analysed using a logistic regression to look for

interaction between time points and serum addition. Analyses were carried out using the TIBCO Spotfire S+ ver 8.2 statistical package (TIBCO Software Inc., Palo Alto, California) by Professor Ian James.

## 2.6. Ethics statement

The study protocol was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee, and informed consent was obtained from all participants in the PDDD cohort. Approval for use of control samples was provided by the Busselton Population Medical Research Institute ethics committee as well as the Red Cross. The conduction of experiments in this thesis using human samples was approved by the Murdoch University Ethics Committee, reference number 2012/058.

# 3. Biomek automation of sample handling, serial dilutions and ELISA protocols

## 3.1. Introduction

The automation of immunological assays, PCR reactions and HLA typing has been rigorously developed by the Institute for Immunology & Infectious Diseases (IIID) at Murdoch University. Automation enables standardization of volume transfer, as well as mixing and tracking of samples through large batch handling of serialized protocols. Automation on robots at IIID utilizes the in-house developed sample tracking program EpiLab. Each sample registered is given a unique medical record number (UMRN) which is used instead of a patient's name. To distinguish between different specimens and samples from different collection dates for the same patient, each sample additionally receives a unique "Base ID", from which samples and their volumes can be tracked through different protocols. To optimize pipetting on Beckman Coulter robots, the BioMek software is used. The program gives a visual representation of the deck layout used on the machine and allows alterations of many different variables, including depth and movement of pipettes, based on lab-ware such as plates or reservoirs. Both EpiLab and BioMek software use comma separated values files (.csv) for tracking of samples and reagents as well as volume identification. Layouts of EpiLab, BioMek software and pipette controls are protocolled in detail in Supplementary Figures S-2, S-3 and S-4.

The Enzyme-Linked Immunosorbent Assay (ELISA) is a fundamental tool in immunological research, and can provide quantitative information on the presence of cytokines, chemokines, molecules or antigen-specific proteins such as antibodies in serum, plasma or supernatant. Final results of the assay are dependent on a number of technical variables that may impact precision if not highly standardized between operators. Large studies often require performance of multiple manual ELISAs which is labour intensive, includes many manual handling steps and is subject to data and sample integrity failure as well as prone to large inter-operator variability. To overcome these difficulties, the ELISA work performed in this thesis, automated

performance of the ELISAs was chosen and included standardized plate coating, sample tracking, sample and reagent addition. All steps in the protocol were optimized and successfully validated.

## 3.2. Results

### 3.2.1. Determination of ideal sample dilution in the commercial EBNA-1 ELISA

Each commercial ELISA was tested according to the manufacturer's instructions, with provided calibrators (pooled human sera; 5, 20, 110 and 200 Arbitrary Units (AU)/ mL) in duplicate, which were used to create a linear standard curve (Figure 3-1). Averages and standard deviations for the calibrators of the commercial ELISAs are summarized in Table 3-1. The manual stated that a sample is considered 'positive' if the optical density (OD) reading is above the highest calibrator, but gives no instructions on how to determine the definitive quantity of antibodies in the sample. Samples can only be quantitatively measured if they fall within the "linear" measuring range of the assay. The standard procedure for quantitating samples above the linear standard curve range is to further dilute samples so the OD of the sample lies within the linear range. Absolute concentrations are then calculated and corrected for the dilution factor. However, this calculation assumes the standard curve continues to be linear above an OD reading of the highest calibrator in the commercial ELISA. Upon performing the suggested 1/100 dilution of 87 MS patient serum samples, 63% and 75% MS samples had OD values too high and out of the linear measuring range for both the EBNA-1 and the VCA ELISAs, respectively.

To serve as the internal control (IC) for all assays, an MS patient serum sample was aliquoted and stored at -80°C until further use. For each assay performed, one fresh aliquot was thawed

and serially diluted 1.4-fold (1/280 to 1/2108). When tested on the commercial ELISA, these serial dilutions resulted in OD values that were not linear, but closely fitted a power curve defined by the equation $y=121.01x^{-0.881}$ ($R^2 = 0.997$) (Figure 3-2; Averages and Standard deviations in Table 3-2). Additionally, serially diluted aliquots from other MS patients were also tested using the same approach, with each producing different slopes (Figure 3-3). We concluded that due to the differences in the power curve slopes, different dilution factors could not be adequately compared from one sample to another as the curves showed different behaviours, and thus could not be compared or expressed as a function (Table 3-3). Taking this into account, a plate of samples in two different dilutions was made in order to determine the ideal dilution for the majority of samples. Dilution at 1:1075 gave the best results within the linear range of the assay and the majority of samples have values above the lower cut-off (specific to each assay) and below the maximum OD (4.0). For subsequent statistical analysis, OD values were transformed (log base 10) to normalize data distribution. Furthermore, the serially diluted IC was additionally used to determine the inter-assay variability between different plates (Supplementary Table S-3)

Sample dilutions were performed in a magMAX Express Microtiter 96 Deep Well Plate (Life Technologies) by diluting 2.8µl of serum with 1536.6µl sample diluent (1/548), followed by serial 1.4-fold dilutions to reach 1/1075. The BioMek FXP had a limited pipetting volume of 500µl, so dilutions 1/768 & 1/1075 required a two-step dilution process:

i) 874µl (2 x 437µl) in 1223.6µl

ii) 723µl (2 x 361.5µl) in 1012.2µl

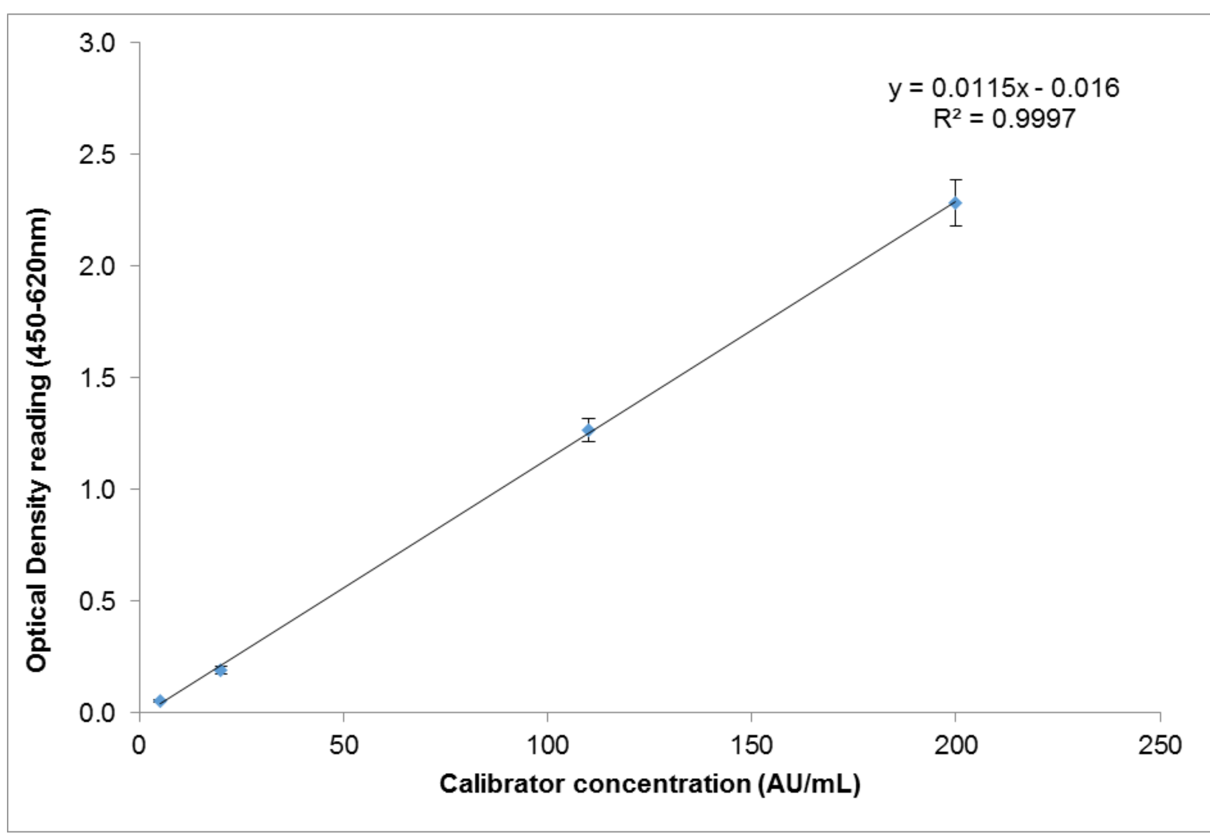


**Figure 3-1. Variability of linear standard curve between 12 commercial EBNA-1 ELISAs.** Linear standard curve of the four anti-EBNA-1 ELISA calibrators (given in arbitrary units (AU): 5, 20, 110 & 200 AU/ mL) measured as optical density (OD) in 12 separate commercial EBNA-1 ELISA plates. Error bars show mean $OD_{450\text{-}620nm}$ ± standard deviation of each calibrator.

**Table 3-1. Optical Density (OD) Average and Standard Deviation of Commercial Calibrators for EBNA-1 and VCA ELISAs (n=12).**

| | EBNA-1 ELISA | | VCA ELISA | |
|---|---|---|---|---|
| **Calibrator Concentration (AU/ml)** | **Average** | **Standard Deviation** | **Average** | **Standard Deviation** |
| **5** | 0.056 | 0.005 | 0.041 | 0.012 |
| **20** | 0.192 | 0.014 | 0.190 | 0.036 |
| **110** | 1.266 | 0.053 | 1.611 | 0.122 |
| **200** | 2.285 | 0.102 | 2.738 | 0.156 |

AU: Arbitrary units. EBNA-1: Epstein-Barr Virus. VCA: Viral capsid antigen.

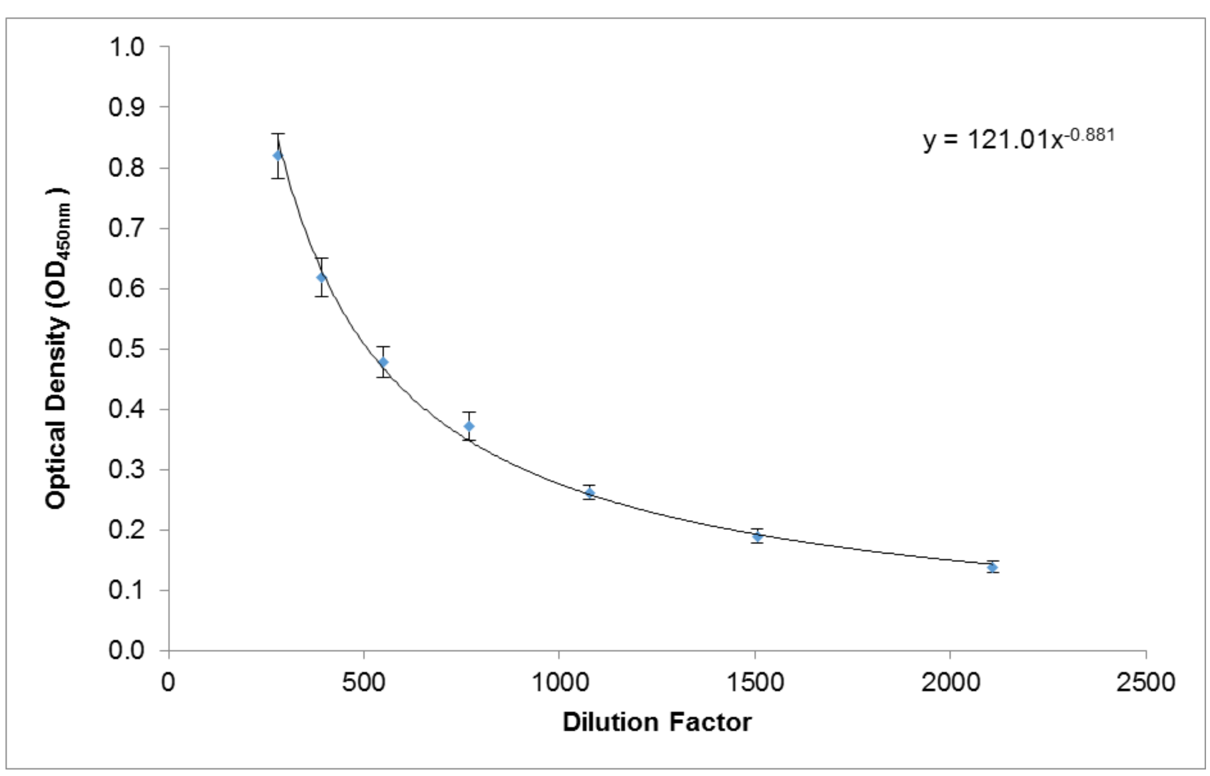


**Figure 3-2. Power curve and inter-assay variability of diluted internal control between 12 commercial EBNA-1 ELISAs.** Error bars show mean Optical Density $(OD)_{450nm}$ ± standard deviation of diluted internal control.

**Table 3-2. Optical Density (OD) Average and Standard Deviation of in-house control serially diluted on commercial EBNA-1 ELISA (n=12).**

| Dilution | Average | Standard Deviation |
|---|---|---|
| **1/280** | 0.820 | 0.037 |
| **1/392** | 0.619 | 0.032 |
| **1/548.8** | 0.478 | 0.025 |
| **1/768.32** | 0.372 | 0.024 |
| **1/1075.648** | 0.262 | 0.012 |
| **1/1505.907** | 0.190 | 0.011 |
| **1/2108.27** | 0.139 | 0.010 |

AU: Arbitrary units. EBNA-1: Epstein-Barr Virus. VCA: Viral capsid antigen.

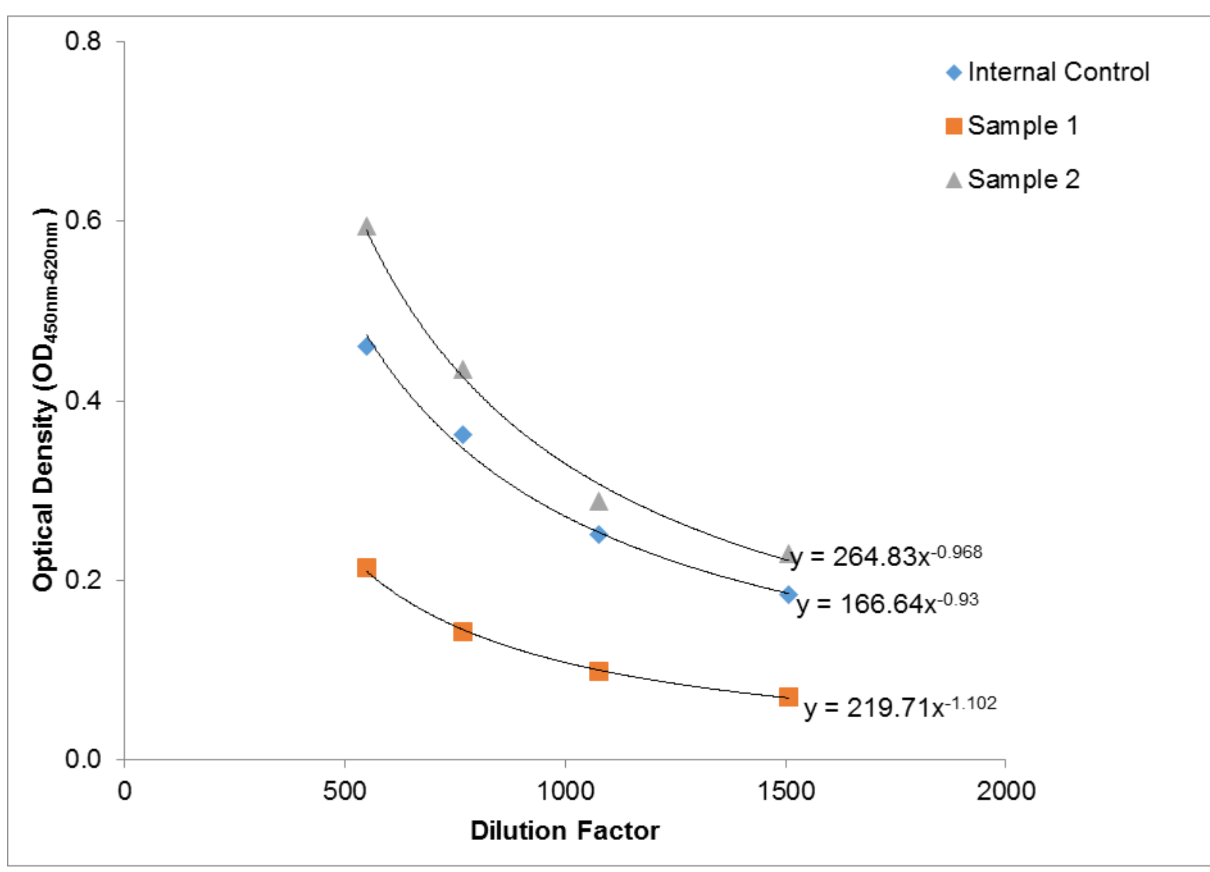


**Figure 3-3. Three samples serially diluted tested with the commercial EBNA-1(long) ELISA resulting in different power curves.** 1.4 fold-serial dilutions of two individual Multiple Sclerosis samples and the internal control sample result curves with different slopes when tested with the commercial EBNA-1 ELISA.

**Table 3-3. Non-linear correlation between two serially diluted MS samples (from Figure 3-3) and the optical density (OD) values from the commercial EBNA-1 ELISA.**

| | **Dilution Factor** | **548** | **768** | **1075** | **1505** |
|---|---|---|---|---|---|
| **MS Sample 1** | OD (450nm) | 0.213 | 0.142 | 0.099 | 0.070 |
| | Difference Ratio Factor | - | 1.50 | 1.44 | 1.41 |
| **MS Sample 2** | OD (450nm) | 0.594 | 0.435 | 0.288 | 0.230 |
| | Difference Ratio Factor | - | 1.37 | 1.51 | 1.25 |

MS: Multiple Sclerosis. OD: Optical Density. EBNA-1: Epstein-Barr Virus Nuclear Antigen-1. ELISA: Enzyme Linked Immunosorbent Assay.

### 3.2.2. Validation of mixing in automated samples

To assure thorough mixing of samples during the dilution process, three aliquots of the IC in duplicate for consistency of results. Samples were serially diluted 1.4-fold (starting with an initial dilution of 1/548). Sample dilutions were mixed on an orbital shaker (30 seconds, 1300rpm) on a BioMek FXP robot, between each dilution step. For analysis, samples were measured for each dilution against the in-house EBNA-$1_{(398\text{-}413)}$ peptide (Figure 3-4) with averages and standard deviations summarized in Table 3-4. The intra-assay variation of the IC for the in-house ELISA was determined to be low (absolute range) and lie between 4.8-23%.

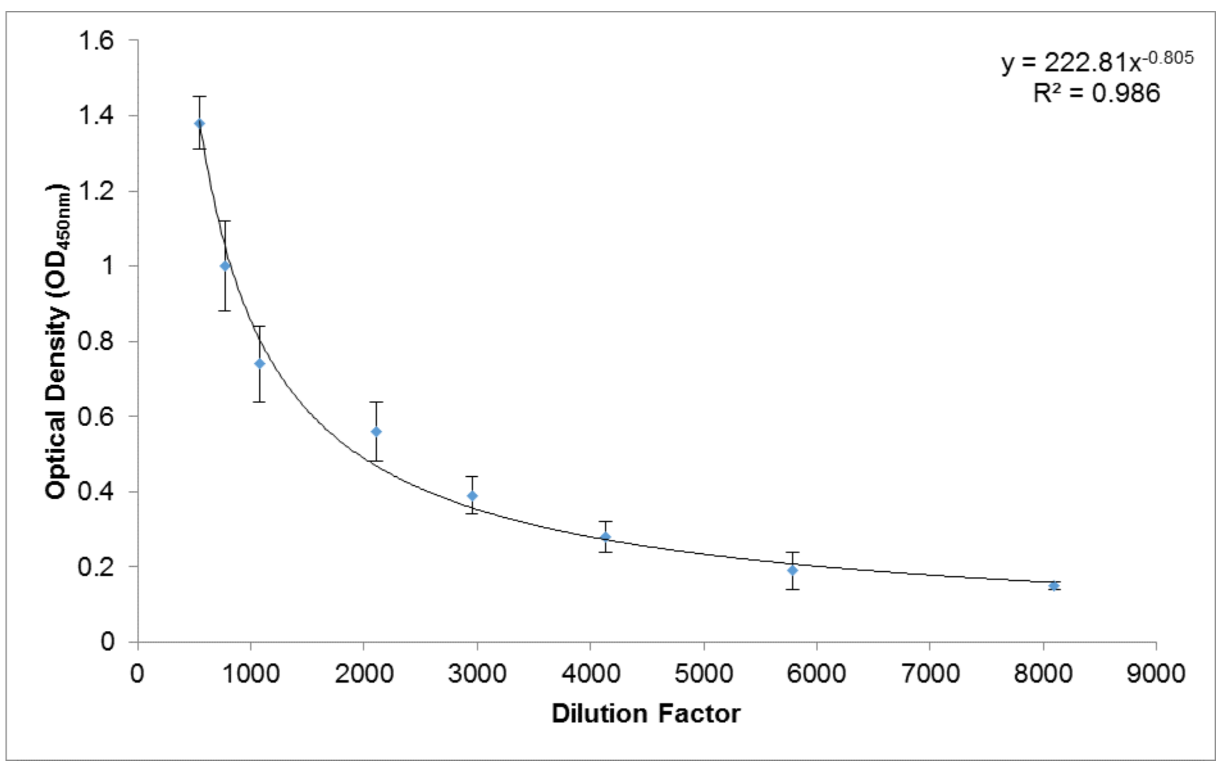


**Figure 3-4. Low intra-assay variability of three individual aliquots of internal control diluted and measured in duplicate, using the anti-EBNA-$1_{(398\text{-}413)}$ in-house ELISA.** Error bars show mean $OD_{450\text{-}620nm}$ ± standard deviation of diluted IC.

**Table 3-4. The Average optical density ($OD_{450-620nm}$) and standard deviations of three aliquots of internal control, diluted and measured in duplicate, using the anti-EBNA-1$_{(398-413)}$ in-house ELISA**

| Dilution | Average | Standard Deviation | %CV |
|---|---|---|---|
| **1/549** | 1.38 | 0.07 | 4.8 |
| **1/768** | 1.00 | 0.12 | 12.17 |
| **1/1076** | 0.74 | 0.10 | 12.92 |
| **1/2108** | 0.56 | 0.08 | 14.97 |
| **1/2952** | 0.39 | 0.05 | 12.2 |
| **1/4132** | 0.28 | 0.04 | 16.02 |
| **1/5785** | 0.19 | 0.05 | 23.46 |
| **1/8099** | 0.15 | 0.01 | 9.91 |

CV: Coefficient of Variability.

## 3.3. Discussion

The quantitation of analytes is beneficial to human health through establishing normal ranges and then comparing with patient cohorts. This can help identify immune environmental changes (such as an increase of pro-inflammatory cytokines) reflective of immune responses. ELISAs continue to be used in a range of immunological applications for large scale epidemiological studies (580) and can be a powerful tool in translational science, as shown for autoantibody detection in RA (630). Benefits of ELISAs include the independence of radioactive materials, which are required for radio-immunoassays, heightened sensitivity in antigen and antibody detection, when compared to older historical methods such as agglutination or immunoelectrophoresis, configuration for a range of different immune targets- and they are generally more cost effective. Ongoing improvement of the quality and utility of ELISA performance as high-throughput assays is increasingly important for studying large patient cohorts. In this chapter we presented a successful and configurable automated system for the performance on an ELISA, incorporating robotics for liquid handling. Limitations of this process include the requirement of “dead volume” of reagents to ensure adequate volume transfer. This means that there is slight waste for some reagents if they cannot be re-used, which additionally reinforces the benefit of combining multiple assays to minimize waste. Benefits of automation include equal reagent use estimation, minimizing the complication of high variability at lower reagent concentrations, reflecting inherent variability of biological assays (631), further compounded by small volumes of patient serum for serial dilutions. Additionally, standardization of assay performance and data security ensured through electronic tracking records of sample transfer and assay results reduced human error in reporting results. Thus, all samples and reagents can be tracked with quality assurance, which is particularly useful for large scale projects, including the cohorts summarized in this study.

# 4. Investigating antibody response against lytic (VCA) and latent (EBNA-1) EBV targets as discriminating factors for Multiple Sclerosis risk

## 4.1. Introduction

MS is a multifactorial disease involving host and environmental risk factors. The strongest genetic contributor is HLA-DR alleles (184, 192), although genetic factors explain less than a third of overall variance (632). Set against this genetic background, EBV infection appears to be an important, and perhaps necessary, step towards the development of MS later in life (559). Epidemiological studies highlighted MS association with IM (456, 457), and many studies have investigated the MS association with serological markers of previous EBV infection in retrospective (567, 633) and prospective studies (341, 545, 565, 574), as well as subsequent meta-analysis (527, 559). Factors including genetics, smoking (269, 568), and vitamin D (580) have been investigated as potential additive or masking risks for EBV antibody responses, with varied levels of interaction reported between publications. A majority of studies, which measured anti-EBNA-1 responses, found significant differences between MS patients and healthy/control cohorts. In addition, anti-EBNA-1 antibodies have been implicated as a diagnostic hallmark of MS with identification of oligoclonal band formation in cerebrospinal fluid (CSF) samples (478, 572) and EBNA-1 specific effector CD4+ T cells capable of cross-reacting with myelin antigens (480). Additionally, Sundström and colleagues (198) found significantly elevated antibodies against a short section of EBNA-$1_{(385-420)}$, an association that was not seen in other overlapping fragments of EBNA-1. This finding has been replicated in studies, which also examined association with HLA (166) and vitamin D levels (580). Mechelli *et al*. (582) investigated antibody responses against EBNA-1 in MS discordant identical twins, where they found elevated antibody responses against a B cell epitope (aa401-411 GRRPFFHPVGE, p=0.006) in the C terminus of EBNA-1 only in affected MS individuals. This B cell epitope is targeted during IM (579), is not HLA restricted, and the study found no association of antibody response against it with smoking or IM history. Additionally, this epitope shares homology with αßC (RRPFF), a candidate autoantigen in MS (634).

Interestingly, Csuka *et al.* (581) found no significance when measuring IgG antibodies against a partially overlapping epitope EBNA-$1_{(398\text{-}404)}$, in MS patients (n=135), suggesting amino acids in position 401-411 are essential. The aim of our study presented in this chapter was to investigate the influence of both poly-specific and epitope-specific anti-EBNA1 and VCA antibodies for MS risk, in a well-characterized Western Australian MS cohort. The following questions were addressed in this chapter:

1. Are there differences in antibody responses against EBNA-1, VCA or EBNA-$1_{(398\text{-}413)}$, both in terms of prevalence of detectable responses and antibody titres between MS patients and controls?
2. Are these antibody responses different when grouped according to gender, age or HLA risk status?
3. Do any of the values correlate with each other, specifically as epitope specific EBNA-$1_{(398\text{-}413)}$ responses are part of the polyclonal anti-EBNA-1 response?
4. Could these factors contribute to a logistic regression model to determine factors influencing MS risk?

## 4.2. Results

### 4.2.1. Study cohort

As expected, the MS cohort was enriched for females compared to the healthy cohort (p<0.001; Table 4-1). The cohorts were well-matched for average age within the different sexes: male cases 49.6 years (SD 11.3), controls 50.8 years (SD 17.7); and female cases 48.3 years (SD 12.0), compared to controls 49.8 years (SD 17.0); overall p=0.4.

**Table 4-1. Demographics of study cohorts.**

| | Healthy Controls | MS Patients | P value |
|---|---|---|---|
| **Number of individuals** | 186 | 426 | - |
| **Gender (M/F)** | 95/91 | 100/326 | p<0.001 |
| **Average Age (mean, SD)** | 50 (17.5) | 48.6 (11.8) | 0.4 |

MS= Multiple Sclerosis, M=male, F=female.

### 4.2.2. HLA-DR allele distribution in cases and controls

We recently determined HLA-DRB1 risk profiles in this cohort, identifying a high-risk group (DRB1*08/*15/*16) as well as protective group/low risk alleles (DRB1*04/*07/*09) (184). Accordingly, cases and controls were classified as "high-risk" if they carried any alleles from the high-risk group, "low-risk" if they carried any protective alleles and no high-risk alleles, or "neutral" if both alleles were outside these groups. Individuals with high-risk HLA-DR alleles were more prevalent among MS cases (odds ratio (OR) 2.40, p=0.0009), and low-risk individuals were less frequent (OR 0.43, p=0.0013), compared with the remaining "neutral" group (Figure 4-1). There was no significant difference in mean ages across the high risk (48.8), neutral (48.5) and low-risk (49.9) groups (p=0.5).

| Risk HLA | Healthy Controls (n=186) | MS (n=426) | P value |
|---|---|---|---|
| Low risk (HLA-DRB1*04/07/09) | 98 | 90 | 0.0013 |
| Neutral risk (HLA-DRB1*01/03/04/10/11/12/13/14) | 35 | 76 | ns |
| High (HLA-DRB1*15/16/08) | 53 | 260 | 0.0009 |

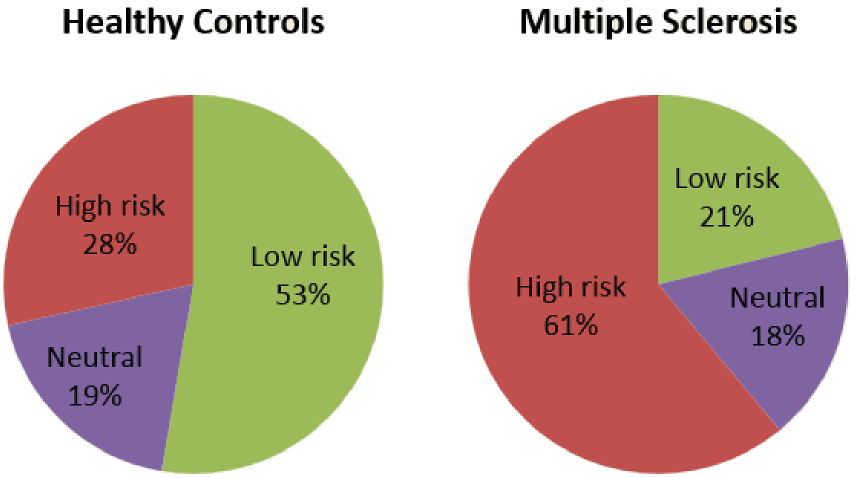


**Figure 4-1. Proportion of high, neutral and low risk HLA alleles in MS and control cohorts.** HLA: Human Leukocyte Antigen. MS: Multiple Sclerosis. ns: non-significant (p>0.05).

#### 4.2.3. Polyspecific EBNA-1 and VCA antibodies in cases and controls

The commercial assays were validated using a serially diluted positive IC (see Chapter 3), which had a low inter-assay variability of 4.4-6.3% for EBNA-1 and 5.3-7.7% for VCA. Significantly more MS individuals had positive (above cut off) values for anti-EBNA-1 IgG antibodies (99.3% vs. 85.5% of healthy controls; $p<10^{-11}$) and anti-VCA IgG antibodies (100% compared to 90.3% of healthy controls; $p<10^{-10}$) (Figure 4-2). The three MS and 27 healthy control serum samples that were initially anti-EBNA-1 negative were tested at a lower dilution (1/100) in order to detect low positive samples. Re-testing of samples resulted in 100% EBV ELISA positivity of MS samples and additional positivity of 15 samples of the 27 healthy controls. When cohorts were combined, there was no significant difference overall in levels of anti-EBNA-1 IgG for males and females (mean difference (MD) 0.044 (Standard Error (SE)

0.041); $p > 0.3$). Importantly, MS cases had significantly higher mean anti-EBNA-1 levels (MD 0.058 (SE 0.042); $p < 10^{-15}$, Figure 4-3B) than healthy controls.

This was also true for anti-VCA IgG titres, where the values in MS cases were higher compared to controls (MD 0.35 (SE 0.035); $p < 10^{-15}$, Figure 4-3A), but they were also higher in females compared to males (MD 0.13 (SE 0.034); p= 0.0001). After adjusting for case/control groups, EBNA-1 antibody levels were significantly increased in the “high-risk” HLA-DR group relative to the “low-risk” group (MD 0.17 (SE 0.045); p=0.0001, Figure 4-3E). Levels in the “neutral” group were marginally higher than the “low-risk” group (MD 0.11 (SE 0.055); p=0.05). There was also no significant difference between antibody levels in the “high-risk” and “neutral” groups (p=0.22). Differences between cases and controls remained highly significant after adjustment for the influence of HLA-DR effects (MD 0.54 (SE 0.042); $p<10^{-15}$, Figure 4-3E), with again no significant difference in EBNA-1 titres in males and females (p=0.24). This was in contrast to observations of anti-VCA antibody levels, with no significant difference between the low and high risk groups (p=0.09), but values were slightly lower in the neutral group compared to the high and low HLA-DR risk groups (Figure 4-3D).

Interestingly, higher EBNA-1 antibody levels were associated with younger age among females (p=0.0009) while values were higher among older males (p=0.02), as illustrated in Figure 4-4. For VCA there was no significant change over age for females (p=0.56), while again for males, values increased with age (p=0.0001). There was no significant difference in the linear slopes among cases and controls (p>0.3).

### 4.2.4. Epitope-specific EBNA-1$_{(398-413)}$ antibodies in cases and controls

We developed and optimised a novel in-house ELISA assay to detect IgG antibodies directed against a previously identified EBNA-1 epitope (166, 480). To confirm the relevance of this epitope sequence *in vivo*, 45 MS samples were successfully amplified and sequenced covering the 16 amino acids of the EBNA-1 epitope PPPGRRPFFHPVGEAD (amino acid code in Supplementary Table S-2). Of these, 40 sequences were completely conserved with a further five samples showing variation at amino acid positions G13 A/V and E14 Q/D/G of EBNA-1.

MS serum samples showed 97.9% positivity against this short peptide, compared with 77.4% healthy controls ($p<10^{-14}$), and as shown in Figure 4-2, with ELISA titres significantly higher among MS cases compared with controls (Figure 4-3C, $p<10^{-15}$). There was no detectable influence of HLA-DR profiles on epitope-specific EBNA-1$_{(398-413)}$ antibody levels in controls (p=0.98, Figure 4-3F), although levels were slightly higher among the high risk group for the MS cases (p=0.0008). For IgG reactivity against EBNA-1$_{(398-413)}$, there was no significant influence of gender (p=0.25, Figure 4-3C) or age (p=0.22, Figure 4-4). Examining correlations between these two anti-EBNA-1 antibody assays, we observed consistently higher epitope-specific antibody levels in cases compared to controls across all anti-EBNA-1 values ($p<10^{-15}$) as shown in Figure 4-5. There were no significant differences in the slopes between cases and controls (p=0.3) nor between sexes (p=0.9). Inter-assay variability of serially diluted IC for this assay was 21.4-25.4%. Additionally, all ELISAs were compared for number of cases and controls that reached maximum optical density (OD 4.0), and while no healthy control samples reached the maximum on any assay, less than 1% of MS cases measured OD 4.0 for EBNA-1 or VCA, but 6.8% of cases reached maximum OD for EBNA-1$_{(398-413)}$ (Table 4-2).

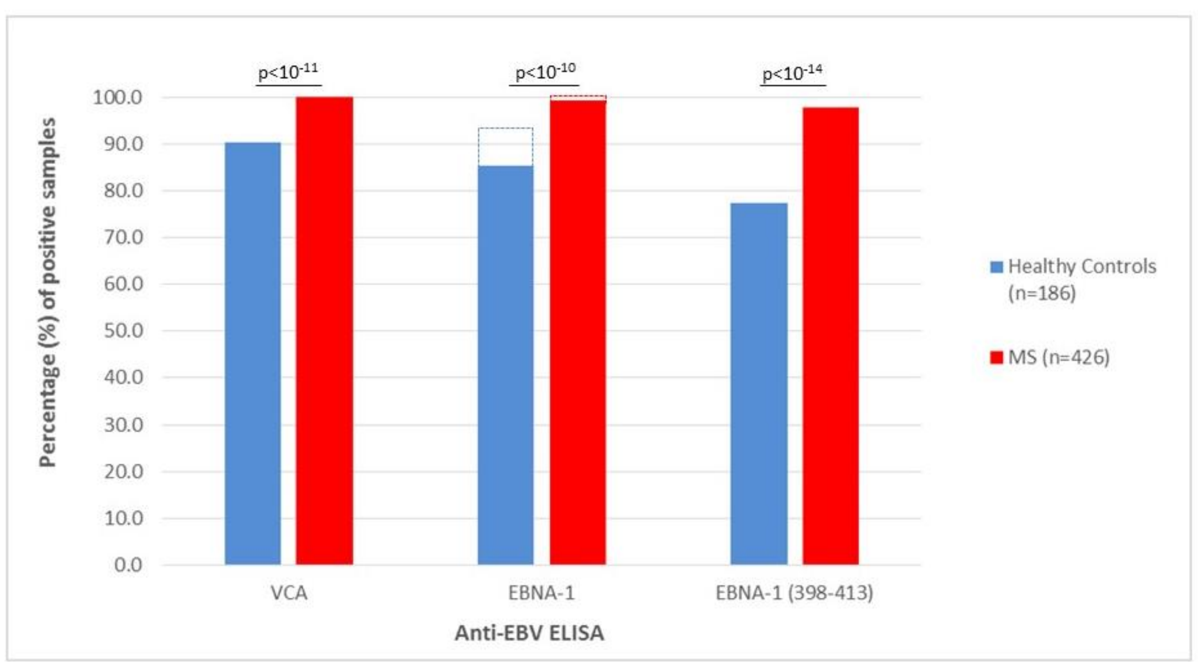


**Figure 4-2. Proportions of positive samples tested by ELISA in Multiple Sclerosis (MS) and healthy control cohorts.** VCA: viral capsid antigen. EBNA-1: Epstein-Barr Virus Nuclear Antigen-1. The MS cohort had significantly more positive anti-EBV antibodies tested by all three assays. Dotted lines above the bars for EBNA-1 show the percentage of samples which tested positive at a lower dilution, showing MS patients were universally EBV positive.

**Table 4-2. Number of samples that reached maximum saturation optical density (OD) for anti-EBV ELISAs.**

| | **Number of samples with maximum OD (4.0)** | |
|---|---|---|
| **Assays** | Healthy Controls (n=186) | MS cases (n=426) |
| **EBNA-1** | 0 | 2 |
| **VCA** | 0 | 4 |
| **EBNA-1$_{(398-413)}$** | 0 | 29 |

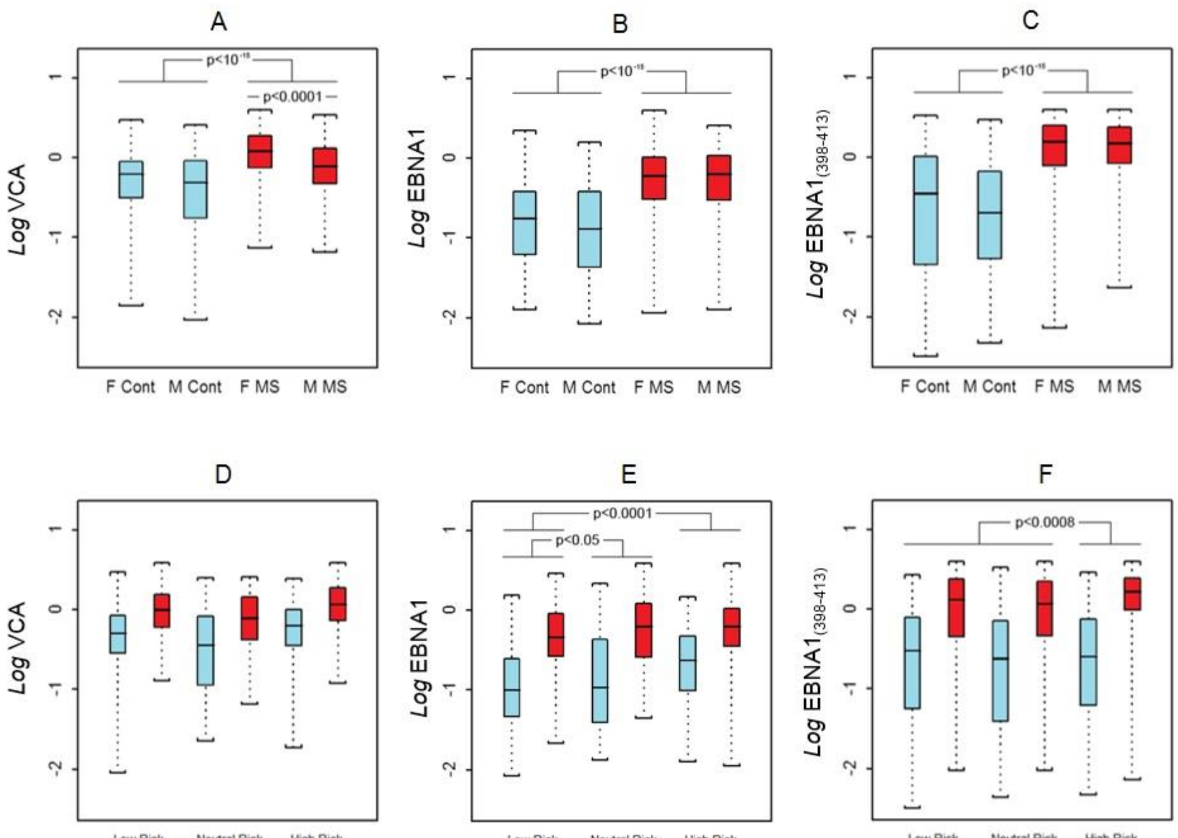


**Figure 4-3. ELISA results for two cohorts stratified by gender and according to HLA-DRB1 allele variation.** Serum levels ($log_{10}$ optical density (OD)) of anti-VCA, anti-EBNA-1 and anti-EBNA-$1_{(398-413)}$ IgG from Multiple Sclerosis (MS; red) cases and healthy controls (Cont; blue) compared by gender (A-C) and HLA-DRB1 risk alleles (D-F). M: male; F: female. VCA: viral capsid antigen. EBNA-1: Epstein-Barr Virus Nuclear Antigen-1. MS: Multiple Sclerosis.

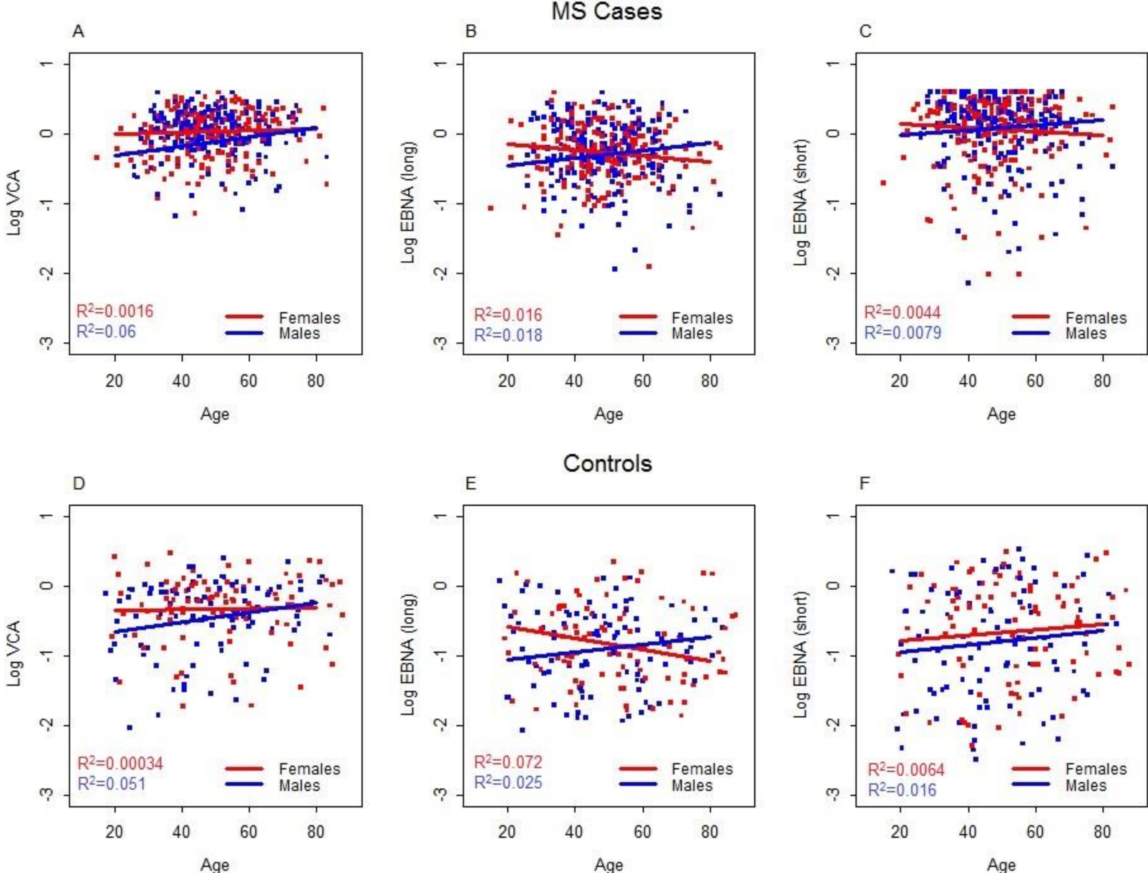


**Figure 4-4. Gender separated correlations of ELISA values and age for Multiple Sclerosis (MS) and Busselton controls.** Correlations between age and serum levels ($log_{10}$ OD) of anti-VCA, anti-EBNA-1 and anti-EBNA-$1_{(398-413)}$ IgG from MS cases (A-C) and Busselton controls (D-F), plotted by gender. There was no difference in slopes between cases and controls ($p>0.05$). Higher EBNA-1 IgG were associated with younger age in females ($p=0.0009$) while values were higher among older males ($p=0.02$). No change in VCA levels over age for females ($p=0.56$), while increased in age for males ($p=0.0001$). No association was seen for age and anti-EBNA-$1_{(398-413)}$ IgG. Overall, there was no significant difference in the linear slopes among cases and controls ($p>0.3$).

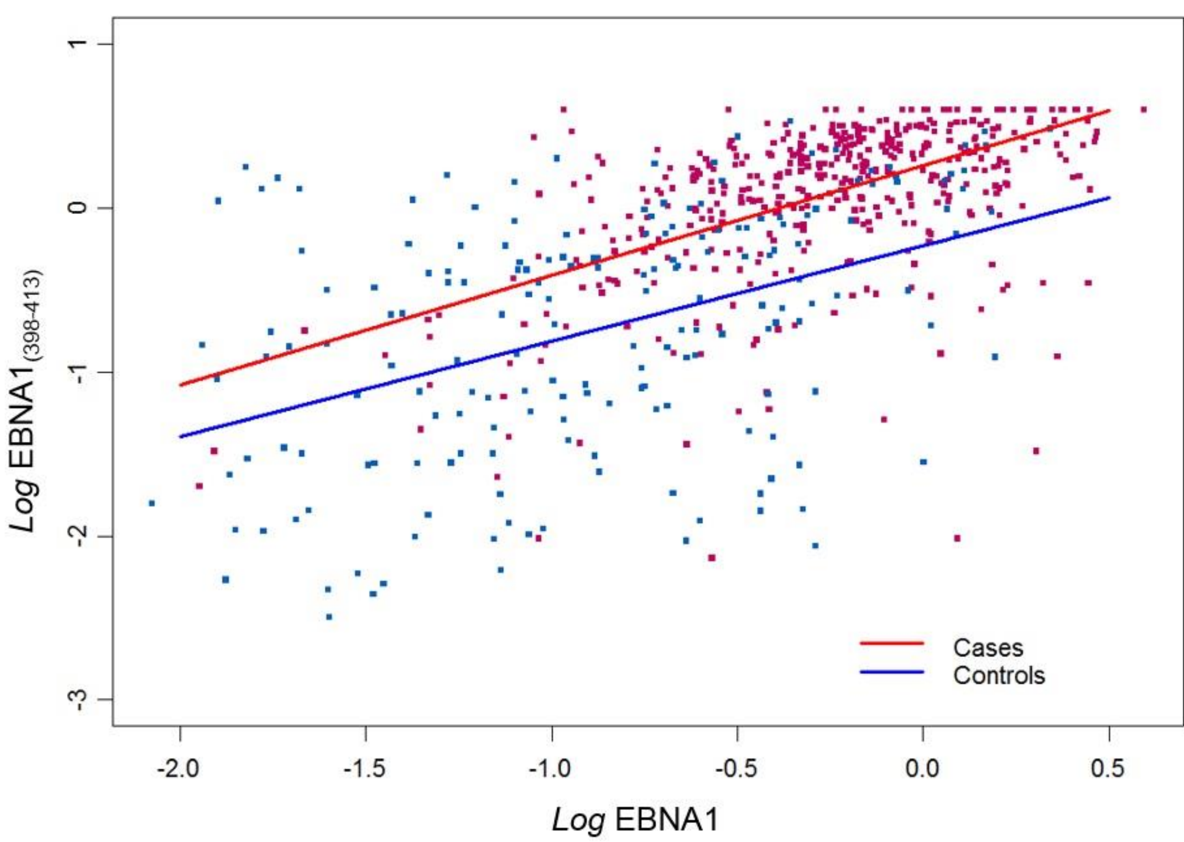


**Figure 4-5. Correlation of two anti-EBNA-1 antibody assays: EBNA-1 and EBNA-1$_{(398-413)}$ targeting polyclonal versus epitope-specific antibodies.** Correlation between anti-EBNA-1 and EBNA-1$_{(398-413)}$ $\log_{10}$ OD values for MS cases (red) and Busselton controls (blue).

### 4.2.5. Logistic regression and receiver-operating characteristic (ROC) curve analyses using predictive models for MS risk

Beginning with a predictive model based on our previous study (184), case-control logistic regression analyses described in Table 4-3 demonstrate the independent effects of high-risk HLA-DR alleles (adjusted OR 2.40, p=0.0009) and protective HLA-DR alleles (adjusted OR 0.43, p=0.0013) as well as female gender (adjusted OR 3.57, $p=1.7\times10^{-10}$). Following incorporation of the significant and independent influences of anti-EBNA-1 values (adjusted OR 6.76 per unit log increase, $p=1.8\times10^{-14}$) and anti-VCA values (adjusted OR 4.96 per unit log increase, $p=6.3\times10^{-7}$) in a second model, the influence of high-risk HLA-DR alleles was no longer significant (adjusted OR 1.40, p=0.26) although the effects of protective HLA-DR alleles (adjusted OR 0.42, p=0.0061) and female gender (adjusted OR 2.63, $p=3.5\times10^{-5}$) were preserved. Finally, in a third model which included the addition of significant EBNA-$1_{(398\text{-}413)}$ values (adjusted OR 3.47 per unit log increase, $p=1.7\times10^{-9}$) significance of anti-EBNA-1 values (OR 3.17, $p=3.0\times10^{-5}$), anti-VCA values (OR 4.30, $p=2.3\times10^{-5}$) as well as protective HLA-DR alleles (OR 0.38, p=0.003) were maintained, but the effect of the high-risk HLA-DR group (OR 1.26, p=0.47) had decreased further. The improvements in predictive ability of the successive models are evident from the receiver-operating characteristic (ROC) curves shown in Figure 4-6 based on the linear-logistic model scores. The final model here is defined by: $2.62 - 0.975(HLA\text{-}DR_{low\text{-}risk}) + 0.23(HLA\text{-}DR_{high\text{-}risk}) - 1.0(Male) + 1.46\times logVCA + 1.15\times logEBNA\text{-}1 + 1.24\times logEBNA\text{-}1_{(398\text{-}413)}$. For this model the area under the curve (C-statistic) is 0.885 (95% confidence interval 0.853-0.911), providing a sensitivity of 394/426=92% and specificity of 119/186=64% at a cut-off logistic value of ≥0 (OR 21.9). These results identify that including commercial anti-EBNA-1 and anti-VCA ELISA values abrogate high risk HLA-DRB1 alleles as a risk factor, as does the addition of in-house anti-EBNA-$1_{(398\text{-}413)}$ ELISA values.

**Table 4-3. Analysis of genetic and serological MS risk factors using progressive logistic regressions.**

| | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** |
| **HLA-DRB1 High-risk group** | 2.40 | 0.0009 | 1.40 | 0.26 | 1.26 | 0.47 |
| **HLA-DR Low risk group** | 0.43 | 0.0013 | 0.42 | 0.0061 | 0.38 | 0.003 |
| **Gender (Female)** | 3.57 | $1.7 \times 10^{-10}$ | 2.63 | $3.5 \times 10^{-5}$ | 2.73 | $4.0 \times 10^{-5}$ |
| **EBNA-1 (commercial) OD values (log)** | | | 6.76 | $1.8 \times 10^{-14}$ | 3.17 | $3.0 \times 10^{-5}$ |
| **VCA (commercial) OD values (log)** | | | 4.96 | $6.3 \times 10^{-7}$ | 4.30 | $2.3 \times 10^{-5}$ |
| **EBNA-1$_{(398\text{-}413)}$ OD values (log)** | | | | | 3.47 | $1.7 \times 10^{-9}$ |

Logistic regressions for significance of MS risk factors identified previously by this group (184) (Model 1), including commercial ELISA values (Model 2) and in-house ELISA results (Model 3).

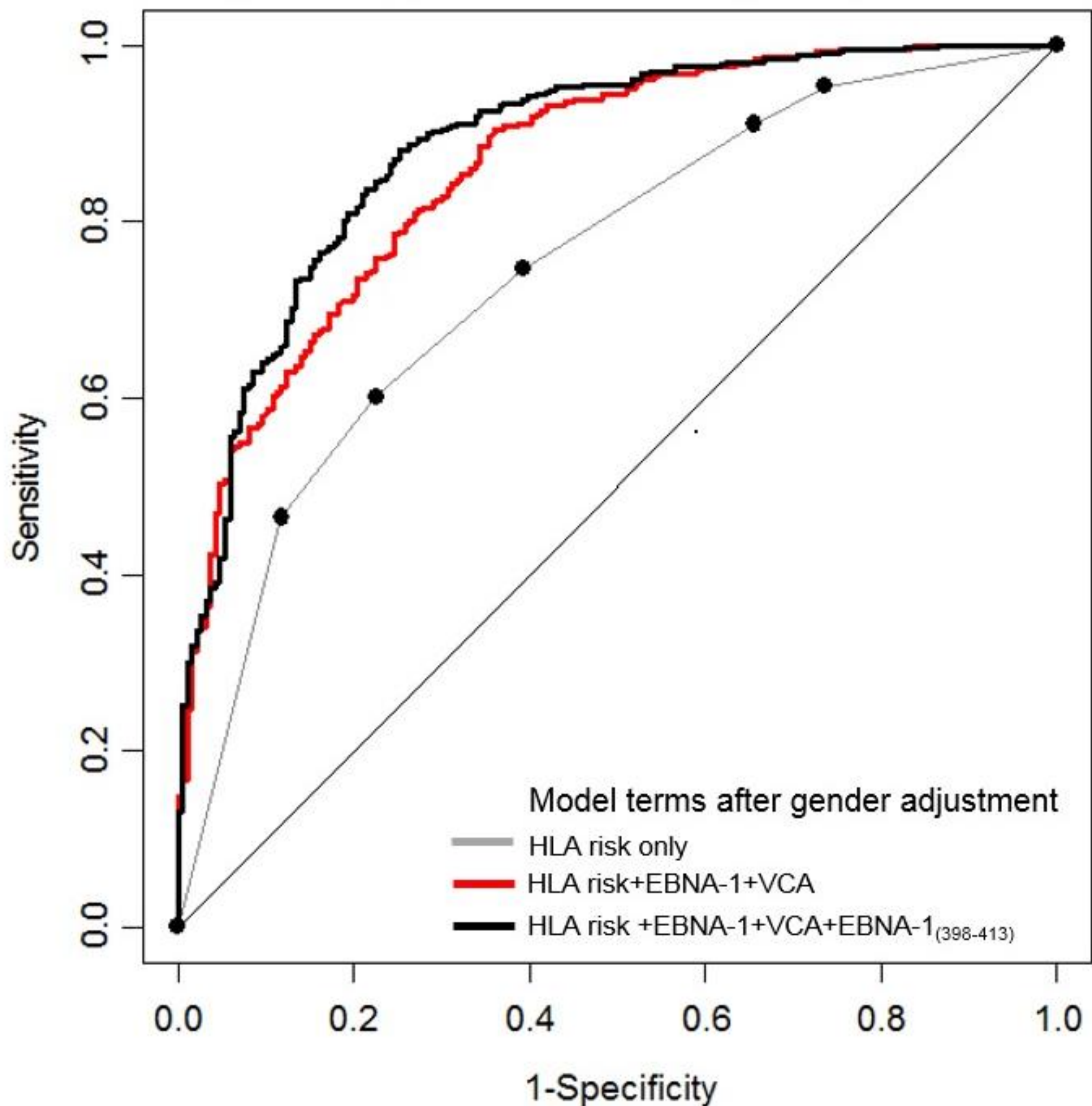


**Figure 4-6. Receiver operating characteristic curve for logistic scores including HLA-DRB1 risk alleles, gender and ELISA values.** Receiver operating characteristic curves demonstrate the additional predictive power of including results from the commercial anti-EBNA-1, anti-VCA and additionally the in-house EBNA-$1_{(398\text{-}413)}$ ELISA compared to the HLA-DR risk groups alone.

## 4.3. Discussion

This is the first EBV-specific serological examination performed using a Western Australian MS cohort as well as using population-based controls, and indeed one of the few based in Australia, with the majority of MS research in the past years coming from Tasmanian groups (259) and Queensland (496). It is also the first population-based study to confirm the specific association between antibody responses directed against a putative B-cell epitope EBNA-1$_{(398-413)}$ and MS risk (166, 582). Both cohorts are largely of European descent, so epidemiological and serological studies may provide insight into the environmental component of MS risk and development in a Southern Hemisphere environment. The MS cohort here was significantly enriched for females at an approximately 3:1 ratio, similar to the global prevalence by gender, however the underlying causative factor for higher risk of MS in females remains unknown.

Utilizing EBNA-1 and VCA specific ELISAs differentiates between latent (past) and lytic (recurrent) infection. Seropositivity for VCA was higher than expected, with almost universal detectable titres in both cohorts, suggesting recurrent lytic infection or a very strong humoural response against VCA in primary infection. The universal seropositivity for EBNA-1 was expected for MS, and seropositivity in 90% of controls fits well within the range reported in other serological studies (449), postulating that EBV infection may be a requirement of MS development. Investigating how many MS patients had IM would be interesting, in particular whether associations seen in American and European studies are also relevant for an Australian cohort. A significant difference for positivity between MS and controls was also detected for EBNA-1$_{(398-413)}$, despite the fact that serological responses were not universally positive among MS cases. Higher antibody titres against EBNA-1, VCA and EBNA-1$_{(398-413)}$ support an EBV-responsive immune environment in MS patients. This study also showed greater statistical

significance against the EBNA-1$_{(398-413)}$ epitope than that seen in previous MS studies investigating antibodies specific for other EBNA-1 regions, such as EBNA-1$_{(385-420)}$ (198), EBNA-1$_{(402-502)}$ (198, 580) and EBNA-1$_{(398-404)}$ (581). Although most of these studies (shown in Figure 4-7) were significant, this study aimed to identify the minimal epitope necessary to reach significance in a large cohort. The significance found suggests that the B cell epitope EBNA-1$_{(398-413)}$, and not just partial regions, is important in MS immune responses.

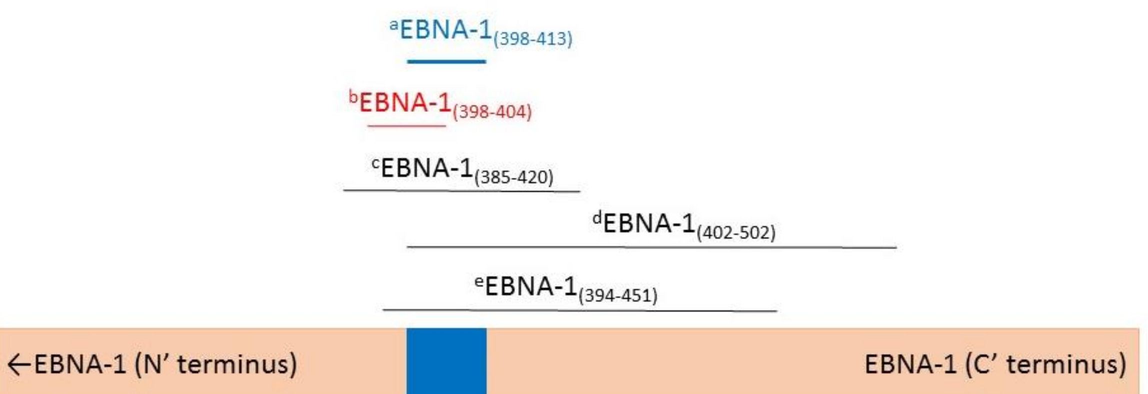


**Figure 4-7. Review of published studies using overlapping epitopes for EBNA-1 (C' terminus) ELISAs.** [a]This study of EBNA-1$_{(398-413)}$ (blue line) compared to other published studies. [b]Red indicates non-significant IgG reactivity (581), while black had significant findings [c](198), [d](198, 580), [e](572). EBNA-1: Epstein Barr Virus Nuclear Antigen-1.

Multiple trends were identified when grouping cases and controls by HLA, gender and age. Elevated anti-EBNA-1 and anti-VCA IgG titres were shown in the "high-risk" HLA group and MS patient compared to controls. Antibody levels against VCA were higher in females (also reported by Mouhieddine *et al* (301)) and older men, and while no significant difference was detected for anti-EBNA-1 IgG between genders, higher levels were found in younger women and older men. It is interesting that we found highly significant influences of age and gender on both EBNA-1 and VCA IgG antibody levels in this study, which would indicate that both values have increased among females relative to males over calendar time – among both cases

and controls. This is intriguing in light of epidemiological evidence that the ratio of females to males with MS has steadily increased in recent decades (111), although any causal association is speculative at present. Unlike anti-EBNA-1 antibodies, anti-EBNA-1$_{(398-413)}$ antibodies were not influenced by HLA-DR genotypes, gender or age – which may in part explain why they remain significantly associated with MS risk even when comparing identical twins (582). This also supports the contention that this EBNA-1 domain is a true B-cell epitope, in keeping with previous studies that demonstrated prominent although transient epitope-specific responses during acute IM (580) as well as dominant epitope-specific anti-EBNA-1 IgG antibodies in healthy controls as well as patients with EBV associated nasopharyngeal carcinoma (635). As observed in previous studies (480, 528, 559, 565, 636), polyclonal anti-EBNA-1 antibody levels that can be measured using commercial ELISA techniques are significantly higher and are highly significantly associated with MS risk. This appears to reflect disease predisposition, given evidence that higher anti-EBNA-1 antibody levels can be observed years before disease onset (565), and remain stably elevated both before and after the onset of clinically isolated demyelinating syndromes (636). Higher anti-EBNA-1 antibody levels have also been associated with increased clinical and radiological features of disease activity beyond the initial demyelinating event (489, 576), although longitudinal anti-EBNA-1 serological profiles have not been assessed in relation to MS disease progression. In this context, it is notable that a recent genome-wide study of determinants of quantitative anti-EBNA-1 antibody levels (406) has identified a large heritable component (~43%) with a much smaller influence of local environment (~4%). Moreover, significant genetic associations were located almost exclusively within the HLA region (406), in keeping with our observations that anti-EBNA-1 antibody levels were associated with HLA-DR alleles in both cases and controls.

There was a significant correlation between polyclonal antibody titres against EBNA-1 and the shorter version of anti-EBNA-$1_{(398\text{-}413)}$, with significant, consistently higher EBNA-$1_{(398\text{-}413)}$ antibody levels in cases to controls across all anti-EBNA-1 values. When comparing the correlated values, there was no significant difference between cohorts or gender, suggesting lack of preferential targeting. However, the consistently "higher titres" of EBNA-$1_{(398\text{-}413)}$ in both cohorts (comparative to EBNA-1) was surprising as it may be reasonable to expect that antibody levels against a small epitope within EBNA-1 would be lower than the polyclonal response against EBNA-1. Accordingly, this may be due to the sensitivity and/or target of the commercial and in-house ELISAs. The commercial ELISA company could not disclose which EBNA-1 targets were used in their antigen pre-coated plates, or how the antigens were produced. Commercially available EBNA-1 proteins often remove the glycine-alanine repeat from the protein due to cross-reactivity, and if used in the commercial ELISA, could exclude the beginning section of EBNA-$1_{(398\text{-}413)}$, explaining differences in reactivity. Several factors including post-translational modifications and whether the EBV targets were produced in cell lines could affect antibody reactivity through epitope conformation. Additionally, the commercial assays used different reagents to the in-house ELISA, which may contribute to different sensitivities, as well as polyclonal antibodies possibly competing for the same target epitope rather than accumulating in their response.

This study extends our previous observation that groups of HLA-DR alleles provide both high-risk and protective influences on MS risk (184), with evidence that the ability to discriminate MS cases and controls can be substantially enhanced by the inclusion of quantitative measures of serological responses specific for Epstein-Barr virus infection. The association of MS and EBNA-$1_{(398\text{-}413)}$ was statistically highly significant, and independent of the broader influence of anti-EBNA-1 antibodies, so that in our final logistic regression model each of these serological

measures contributed greater than 3-fold increased adjusted OR per unit increase in $log_{10}$ OD value (Table 4-2). Incorporating the combined effects of HLA-DR genotyping and quantitative anti-Epstein-Barr virus antibody levels provides for a final logistic score that performs remarkably well in terms of diagnostic sensitivity and specificity, as shown in Figure 4-6, given that neither of these parameters has any direct association with the neurological features of MS. Previous studies have identified statistical interactions between HLA-DRB1*15 and anti-EBNA-1 antibody levels (166) or history of EBV-associated IM (458, 637), suggesting that these risk factors may share a common pathway in disease susceptibility. Our data would support this view, particularly the finding that the strong independent effect of high-risk HLA-DR alleles (model 1, p=0.0009) on MS risk (Table 4-4) was substantially abrogated after incorporation of the influence of anti-EBNA-1 antibody levels (model 2, ($EBNA\text{-}1_{(long)}$, $p=1.8\times10^{-14}$; high-risk HLA-DR alleles, p=0.26). These results require cautious interpretation when considering that this cohort has served a 'discovery' dataset, and it is clear from other studies that have estimated the influence of genetic risk factors in MS (632) that the classification sensitivity and specificity will typically be lower when examined in a validation dataset. Ideally, to test the robustness of the risk model analysis, the in-house ELISA would be validated on a separate MS cohort, either from Australia or the Northern Hemisphere. This could be of use for other studies of MS cohorts to utilize grouping of HLA-DR risk alleles and to incorporate anti-EBNA-1 antibody responses. Nevertheless, it is interesting that our own estimates of the influence of HLA-DR genotypes closely match those identified by another study (632). The discriminatory capacity of our final model, with a sensitivity of 92%, specificity of 64% and an overall odds ratio of 21.9 for a logistic score greater than zero, certainly argues for the relevance of Epstein-Barr virus-specific immunity in MS pathogenesis, as well as for the potential development of diagnostic and treatment strategies that specifically target this aspect of disease susceptibility. Limitations of this study include the commercial

assays providing arbitrary calibrators for quantifying antibody levels. Additionally, the manufacturer suggestion of diluting all samples 1/100 results in a majority of samples reaching maximum OD and requiring re-testing. This study combated the limitation by finding the dilution 1/1075 for most samples to fall within the linear range of the assay (Figure 4-2 and Table 4-2), where few samples reach maximum OD (Table 4-2) and re-testing of negative samples showed a minor increase of seropositivity (Figure 4-2). Our anti-EBNA-1$_{(398\text{-}413)}$ ELISA had the highest number of cases reaching maximum OD, however this should reflect the sensitivity of the in-house assay.

## 4.4. Summary

Results from this study suggest that in addition to the significant role of HLA-DR risk alleles, the ability to discriminate MS cases from controls can be substantially enhanced by the inclusion of quantitative measures of the serological response to Epstein-Barr Virus infection. Specifically, MS patients had significantly higher IgG antibody responses against both EBNA-1 and VCA. When further investigating IgG antibody responses against EBNA-1, epitope responses against a short EBNA-1$_{(398\text{-}413)}$ epitope were additionally elevated in MS patients compared to controls. Importantly, the MS cohort proved to be universally positive for previous EBV infection compared to 90% of controls. Unlike antibody response against EBNA-1, the anti-EBNA-1$_{(398\text{-}413)}$ antibodies were not influenced by HLA-DR genotypes, gender or age, which may in part explain why they remain significantly associated with MS risk even when comparing identical twins (582). Incorporating the combined effects of HLA-DR genotyping and quantitative anti-EBV IgG antibody levels provides a final logistic score that performs remarkably well in terms of diagnostic sensitivity and specificity, given that neither of these parameters has any direct association with the neurological features of MS.

# 5. Citrullination in MS pathogenesis: antibody responses against a citrullinated B cell epitope target

## 5.1. Introduction

### 5.1.1. Chemical process of citrullination

Citrullination is a post-translational process in which arginine is converted to the non-standard amino acid citrulline (638). This deimination is facilitated by the enzyme family peptidylarginine deiminase (PAD) (639) as shown in Figure 5-1. It has been shown that the change in charge alters the secondary and tertiary structure of the protein (640, 641) and is implicated in controlling and altering gene expression (642, 643), but the openness of protein structure can make it more susceptible to enzymatic proteolysis.

**Figure 5-1**. **Process of citrullination.** Unedited from (644).

### 5.1.2. Citrullination in autoimmune diseases

Citrullination has been documented in other neurological disorders including Alzheimer's and Creutzfeldt–Jakob disease (645, 646) but most prominently in RA, where anti-citrullinated autoantibodies have provided strong diagnostic value and are commonly used in clinical practice (647). In RA, antibodies specific for citrullinated EBNA-1$_{(35-85)}$ showed diagnostic specificity of 98.5% and correlated with other disease-specific antibodies against fibrinogen in a recent study (648). Elevated antibodies against EBNA-1 have also been reported (649), and protein citrullination alters the protein presentation by HLA-DR molecules (650, 651) and

subsequent T cell responses (170, 179, 652, 653). Also in RA, single nucleotide polymorphisms (SNPs) have been reported that can cause increased expression of PAD genes, causing more citrullination (654).

### 5.1.3. Citrullination in MS

Citrullination can target multiple proteins including MBP, glial fibrillary acidic protein (GFAP), nuclear proteins and members of the CXC chemokine family (655-658), which play a role in immune cell recruitment and migration into the CNS. MBP is a component of myelin that sheaths and protects nerves in the brain and spinal cord, is heavily citrullinated in children, but decreases over years of brain development. The enrichment of citrullinated MBP and GFAP (659) in MS brain lesions raises the possibility that this modified autoantigen could be a candidate target. Citrullination of MBP causes its open structure to be degraded more easily by Cathepsin D (639, 660, 661), which can be produced by macrophages and reactive astrocytes (498, 662). Susceptibility to degradation is correlated to the amount of citrulline within the MBP (641, 660, 661). Citrullination causes partial unfolding, leading to less tight packing of myelin sheaths and therefore to destabilization (663-665). This could potentially lead to exposure of additional epitopes, subsequently targeted by cross-reactive immune cells (641, 666). Bradford *et al.* (659) suggested citrullination could occur by cell death (extracellularly) or during myelin degradation from phagocytosis (intracellularly). Numerous studies have investigated citrullination of myelin and neural proteins in humans and mouse models (Table 5-1), but no reports to date have investigated correlation of citrullination levels or immune responses to citrulline and MS HLA risk markers. In MS cases, levels of citrullination and antibodies against citrullinated MBP are elevated (667). It is currently under debate whether immune responses to these citrullinated MBP epitopes could explain Th1 polarization (increased inflammatory cytokine production) in MS patients (668) or whether the

modifications of a usually identified 'self-peptide' allows presentation of new epitopes to the immune system (667). These concepts need to incorporate the fact that citrulline and antibodies targeting modified proteins occur naturally within a healthy immune repertoire (669), as do MBP-reactive T cells (246, 670). However, the gradual and consistent change in auto-reactivity regulation could develop into an immune reactivity leading to pathologic conditions (671, 672).

**Table 5-1. Summary of investigations on citrullination in human and Experimental Autoimmune Encephalomyelitis (EAE) models of MS.**

| Human | Mouse (EAE) model |
|---|---|
| **↑ Citrulline protein in brain matter (673-680)** | ↑ Citrullination in post mortem brain (675, 676, 680) |
| **↑ Citrullinated MBP in brain matter (673, 674, 677, 678)** | ↑ PAD2 and PAD4 in synovial and brain tissue (642, 675) |
| **↑ PAD4 (675, 677)** | ↑ Citrullinated GFAP in EAE than OND (681, 682) |
| **↑ PAD2 (674)** | MBP and GFAP citrullinated in MOG-induced EAE (681) |
| **↑ Nuclear histone citrullination (675)** | PAD2 K/O mice citrullinated MBP with PAD4 (686) |
| **↑ Citrullinated GFAP in SPMS than other neurological disorders (OND) (681, 682)** | Hypercitrullination of CNS proteins, correlating to demyelination in EAE (680) |
| **↑ Proteinases in MS CSF (683)** | Citrulline proteins highest in relapse phase of EAE, compared to acute-phase; increased citrulline associated with myelination during development of CNS (660) |
| **↑IgG, IgM & IgA against MBP, MOG and/or αß-C. Adding these antibodies to lymphocytes caused blast formation and pro-inflammatory cytokines. (684)** | ↑ PAD2 expression had more severe course (687) |
| **Anti-citrullinated MBP antibody response predicts CIS conversion to MS (667, 685)** | |

MBP: myelin basic protein. PAD: peptidylarginine deiminase. K/O: knockout. GFAP: glial fibrillary protein. SPMS: secondary progressive MS. OND: other neurological disorders. MOG: myelin oligodendrocytic protein. CIS: clinically isolated symptoms.

### 5.1.4. Viral target citrullination and role in MS

The diagnostic value of antibodies against citrullinated proteins has demonstrated importance and value in RA (688). Although understanding the role of citrullination of MBP and its

consequences in disease pathogenesis is important, our work is focused on the persistent and potentially damaging immune responses to latent EBV infection. Notably, the B cell epitope EBNA-1$_{(398-413)}$ discussed in Chapter 4 shares homology (aa 405-9) with alpha beta crystalline (αß-C; Table 5-2), a small heat shock protein which is also an autoantigen candidate for MS.

**Table 5-2. Amino acid sequence comparison of ELISA peptides.**

| Peptide | Amino acid sequences |
|---|---|
| **EBNA-1$_{(398-413)}$** | $^{aa398}$PPPG**RR**PFFHPVGEAD$^{aa413}$ |
| **EBNA-1$_{(398-413\ CIT)}$** | PPPG**CitCit**PFFHPVGEAD |
| **Alpha Beta Crystallin (αßC)** | **RR**PFF |

Homology shared between EBNA-1 epitope and αßC underlined.

Antibodies against αß-C are part of a healthy immune repertoire but have also been shown to accumulate in the oligodendrocyte-myelin unit in early MS lesions (689). αß-C protein has been identified in MS CSF (690), and associated with other neurological disorders (691-693). αß-C was originally detected in optical lenses but has since been also identified in kidneys, heart, skeletal tissue and CNS (600, 690, 691, 694-697), but not in spleen, liver or lymphoid tissue (600, 691, 695-697). However, Bruno *et al* (698) did report αß-C in lymphoid tissue, and this tissue has also been reported to contain MBP (699). Stoevring *et al* (690) suggested αß-C produced by oligodendrocytes is incorporated extracellularly into myelin sheaths, accessible to be presented by the MHC class II pathway, leading to an inflammatory CD4+ T cell response. So far it is unclear whether this pathway is MS-specific as the protein and antibodies are present in healthy individuals also without causing comparable symptoms. Our study aims to identify whether antibody responses can be measured against a citrullinated form of the EBNA-1$_{(398-413)}$ peptide, and if so how this contributes to the MS risk model described in Chapter 4.

In order to address the influence of citrullination of EBNA-$_{1(398\text{-}413)}$ as a risk factor in MS, the study described in this chapter used the same serum samples from diagnosed MS patients from the Perth Demyelinating Disease Database (PDDD) and control cohort from the Busselton Study as described in Chapter 4. The following questions were addressed in this chapter:

1. Are there any differences for antibody responses, in terms of positivity and titres, for EBNA-$1_{(398\text{-}413\ CIT)}$ seen between MS cases and healthy controls?
2. Do these values differ according to gender, age or HLA risk status?
3. What is the correlation between anti-EBNA-$1_{(398\text{-}413)}$ and its citrullinated version EBNA-$1_{(398\text{-}413\ CIT)}$?
4. Could incorporation of antibody levels against the citrullinated version of EBNA-$1_{(398\text{-}413)}$ improve a logistic regression model to determine risk for MS development?

## 5.2. Results

### 5.2.1. Epitope-specific anti-EBNA-1$_{(398\text{-}413\ CIT)}$ antibodies in cases and controls

Among MS serum samples, 82.8% were found to have positive antibody responses against EBNA-1$_{(398\text{-}413\ CIT)}$, compared with 40.3% of healthy controls ($p<0.00005$), and as shown in Figure 5-2, ELISA titres were significantly higher among MS cases compared with controls ($p<0.00005$). There was no detectable influence of HLA-DR profiles (Figure 5-3), gender or age on epitope-specific EBNA-1 antibody levels in controls or MS samples ($p>0.3$). Seven MS samples and no healthy controls reached the maximum optical density (OD 4.0) using the EBNA-1$_{(398\text{-}413\ CIT)}$ ELISA (Table 5-3). A correlation can be seen for IgG antibodies against citrullinated and uncitrullinated targets in both cohorts (Figure 5-4), but a trend is more apparent through reviewing IgG levels as a ratio of EBNA-1$_{(398\text{-}413\ CIT)}$/EBNA-1$_{(398\text{-}413)}$ compared with EBNA-1$_{(398\text{-}413)}$ (Figure 5-5). Here, both cohorts show EBNA-1$_{(398\text{-}413\ CIT)}$ antibody levels proportional to anti-EBNA-1$_{(398\text{-}413)}$, with EBNA-1$_{(398\text{-}413\ CIT)}$ values rarely being higher than antibodies against the uncitrullinated epitope. For the few samples that had comparatively higher EBNA-1$_{(398\text{-}413\ CIT)}$ values, the proportion of these samples were significantly higher in the MS cohort (Table 5-4, $p<0.05$). Inter-assay variability of serially diluted IC for this citrullinated target ELISA assay was comparable to the uncitrullinated ELISA ranging from 20.9 to 28.1%.

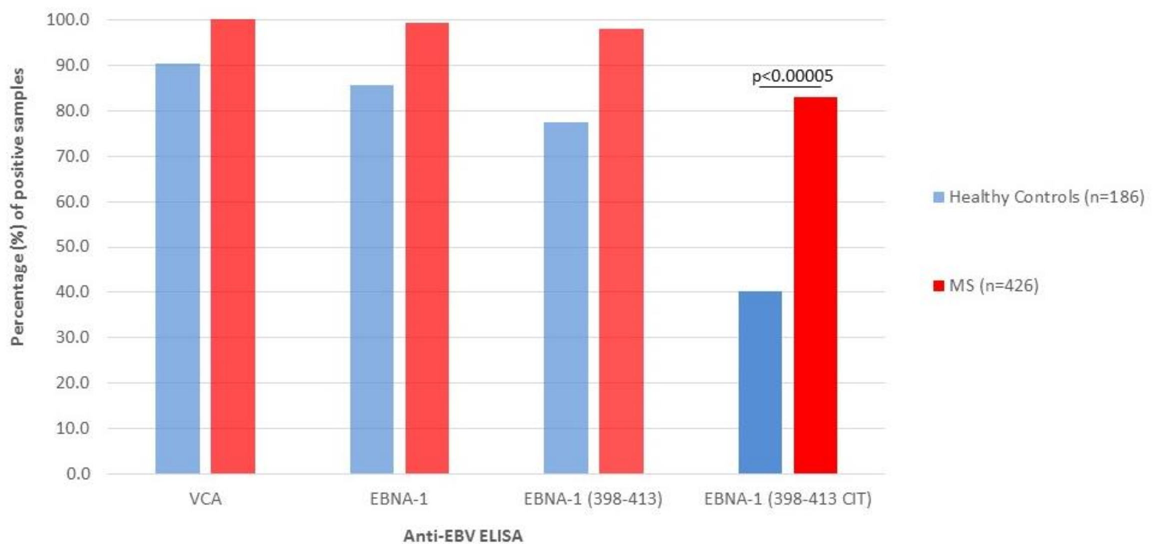


**Figure 5-2. Seropositivity of samples against EBNA-1$_{(398\text{-}413\ CIT)}$, compared to past ELISAs.** VCA: viral capsid antigen. EBNA-1: Epstein-Barr Virus Nuclear Antigen-1. MS: Multiple Sclerosis. The MS cohort had significantly more positive anti-EBV antibodies tested by the EBNA-1$_{(398\text{-}413\ CIT)}$ ELISA.

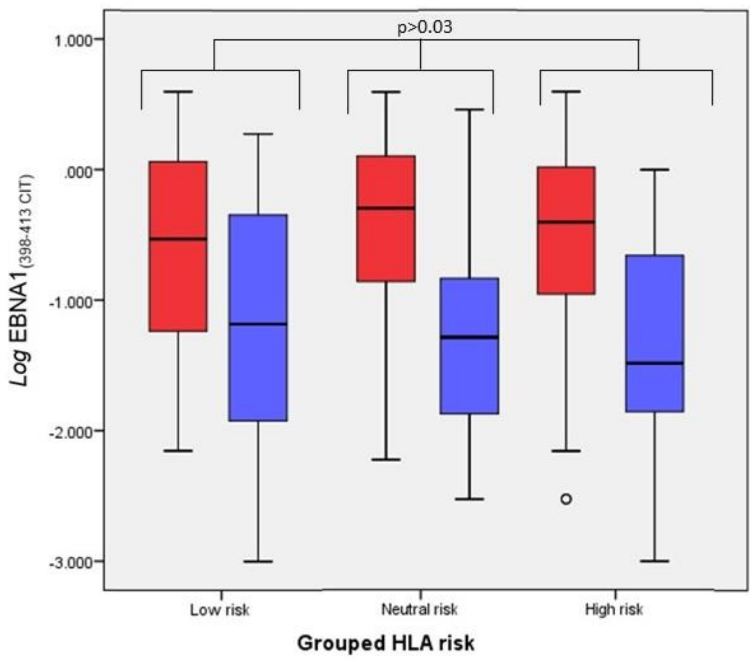


**Figure 5-3. Anti-EBNA-1$_{(398\text{-}413\ CIT)}$ IgG levels between cohorts stratified by HLA-DRB1 allele variation.** Serum levels ($log_{10}$ OD) from MS (red) cases and healthy controls (blue). MS anti-EBNA-1$_{(398\text{-}413\ CIT)}$ IgG are significantly higher compared to controls for each HLA risk group (low, neutral and high), but no significant difference was seen between the HLA risk groups for either cohort.

**Table 5-3. Number of samples that reached maximum saturation optical density (OD) for anti-EBNA-1 in-house ELISAs.**

| | Number of samples with maximum OD (4.0) | |
|---|---|---|
| **Assays** | Healthy Controls (n=186) | MS cases (n=426) |
| **EBNA-1$_{(398\text{-}413)}$** | 0 | 29 |
| **EBNA-1$_{(398\text{-}413\ CIT)}$** | 0 | 7 |

The seven MS samples that measured maximum OD for EBNA-1$_{(398\text{-}413\ CIT)}$ were also maximum OD for the EBNA-1$_{(398\text{-}413)}$ ELISA.

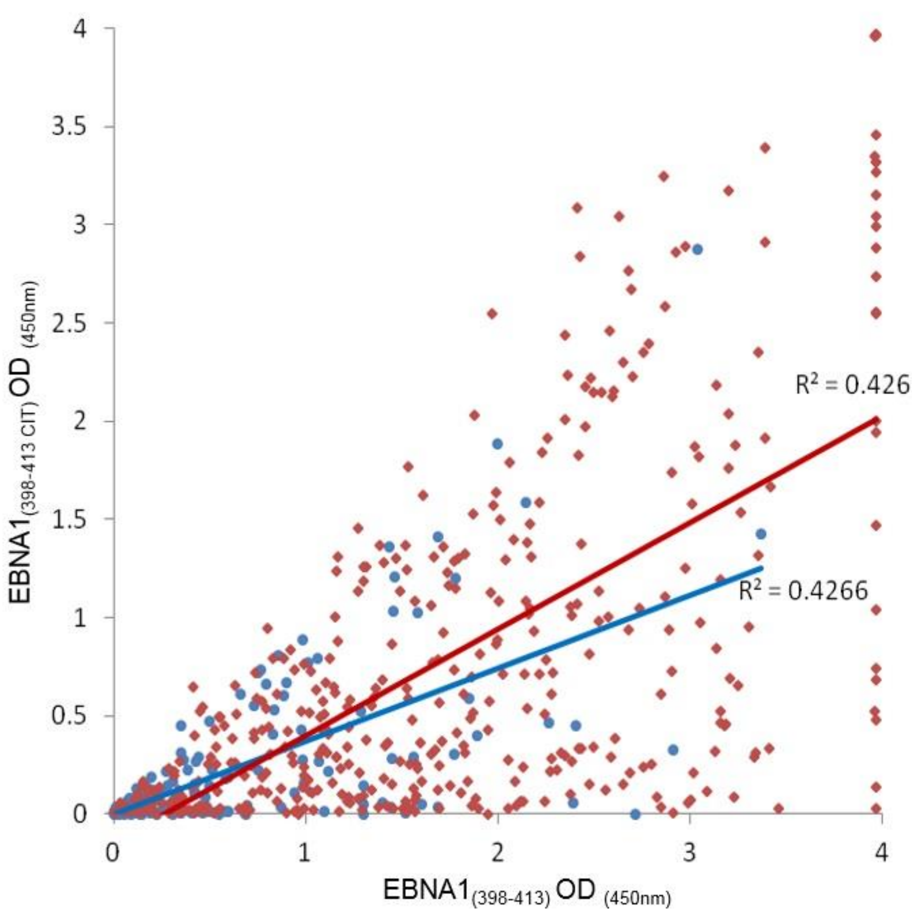


**Figure 5-4. Correlation of optical density (OD) reflecting EBNA-1$_{(398\text{-}413)}$ and EBNA-1$_{(398\text{-}413\ CIT)}$ specific IgG levels.** Both MS cases (red) and healthy controls (blue) show moderate correlation between assays. Anti-EBNA-1$_{(398\text{-}413\ CIT)}$ IgG levels in all samples appear to consistently have a smaller OD than anti-EBNA-1$_{(398\text{-}413)}$ IgG, with increased variability of this at >OD 3.0.

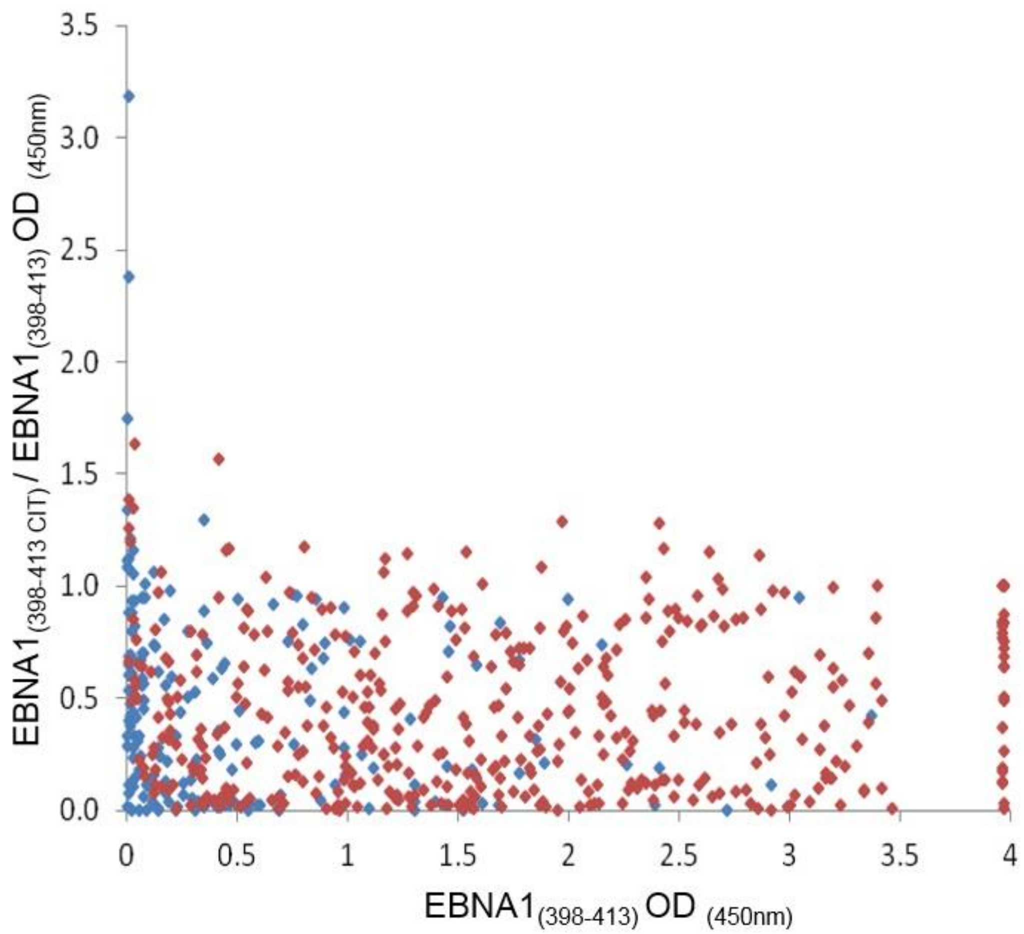


**Figure 5-5. Ratio of EBNA-1$_{(398\text{-}413\ CIT)}$/EBNA-1$_{(398\text{-}413)}$ to EBNA-1$_{(398\text{-}413)}$ from epitope-specific antibody assays.** MS cases (red) and healthy controls (blue) show a maintained ratio between cases and controls for comparing antibody levels on the two assays. Both cohorts show a majority of samples have EBNA-1$_{(398\text{-}413\ CIT)}$ levels proportional to EBNA-1$_{(398\text{-}413)}$.

**Table 5-4. Number of samples with higher antibodies against EBNA-1$_{(398\text{-}413\ CIT)}$ than EBNA-1$_{(398\text{-}413)}$.**

| **Number of samples with EBNA-1$_{(398\text{-}413\ CIT)}$ > EBNA-1$_{(398\text{-}413)}$** | | |
|---|---|---|
| Healthy Controls (n=186) | MS cases (n=426) | |
| 2 (1%) | 20 (4.7%) | $p<0.05$ |

A larger proportion of MS samples had higher EBNA-1$_{(398\text{-}413\ CIT)}$ values compared to EBNA-1$_{(398\text{-}413)}$ ELISA.

### 5.2.2. Logistic regression and receiver-operating characteristic curve analyses

As previously described within Chapter 4, measurement of EBNA-1-specific IgG antibody levels significantly improved the ROC curve in case-control logistic regression. Model 4 (Table 5-5) included the addition of significant EBNA-$1_{(398\text{-}413\ CIT)}$ values (adjusted OR 1.74 per unit log increase, $p=1.59\times10^{-3}$) which slightly reduced but maintained the significant influence of anti-EBNA-$1_{(long)}$ values (OR 2.92, $p=1.42 \times 10^{-4}$), anti-VCA values (OR 4.26, $p=3.19\times10^{-5}$) as well as protective HLA-DR alleles (OR 0.40, $p=5.60\times10^{-3}$). However, as with the inclusion of EBNA-$1_{(398\text{-}413)}$ in Model 3, the high-risk HLA-DR group remained non-significant (OR 1.42, p=0.47). The inclusion of EBNA-$1_{(398\text{-}413\ CIT)}$ significantly contributed to the model, however the addition was shown to decrease the significance of EBNA-$1_{(398\text{-}413)}$. Therefore, the addition of this anti-EBNA-$1_{(398\text{-}413\ CIT)}$ assay shows minimal contribution to improving the overall logistic regression model, as evident from the ROC curves shown in Figure 5-6 based on the linear-logistic model scores. With the addition of the citrullinated EBNA-1 peptide ELISA, the final model is defined by $2.90 - 0.922(HLA\text{-}DR_{low\text{-}risk}) + 0.35(HLA\text{-}DR(_{high\text{-}risk}) - 0.99(Male) + 1.45\times logVCA + 1.07\times logEBNA_{(long)} + 0.79\times logEBNA_{(398\text{-}413)} + 0.55\times logEBNA_{(398\text{-}413\ CIT)}$. This model provides a sensitivity of 391/426=92% and specificity of 121/186=65% at a cut-off logistic value of ≥0 (OR 20.8). This, in fact, reduces the discrimination capability in model 3 that was described in Chapter 4.

**Table 5-5. Analysis of genetic and serological Multiple Sclerosis risk factors using progressive logistic regression models.**

| | Model 2 | | Model 3 | | Model 4 | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** |
| **HLA-DR High risk group** | 1.40 | 0.26 | 1.26 | 0.47 | 1.42 | 0.29 |
| **HLA-DR Low risk group** | 0.42 | 0.0061 | 0.38 | 0.003 | 0.40 | $5.60 \times 10^{-3}$ |
| **Gender (Female)** | 2.63 | $3.5\times10^{-5}$ | 2.73 | $4.0\times10^{-5}$ | 0.37 | $6.04 \times 10^{-5}$ |
| **EBNA-1$_{(long)}$ OD values (log)** | 6.76 | $1.8\times10^{-14}$ | 3.17 | $3.0\times10^{-5}$ | 2.92 | $1.42 \times 10^{-4}$ |
| **VCA OD values (log)** | 4.96 | $6.3 \times 10^{-7}$ | 4.30 | $2.3 \times 10^{-5}$ | 4.26 | $3.19 \times 10^{-5}$ |
| **EBNA-1$_{(398\text{-}413)}$ OD values (log)** | | | 3.47 | $1.7 \times 10^{-9}$ | 2.20 | $1.62 \times 10^{-3}$ |
| **EBNA-1$_{(398\text{-}413\ CIT)}$ OD values (log)** | | | | | 1.74 | $6.4 \times 10^{-3}$ |

Logistic regression models for MS risk factors identified previously by this group including commercial ELISA values (Model 2), EBNA-1$_{(398\text{-}413)}$ results (Model 3) and EBNA-1$_{(398\text{-}413\ CIT)}$ (Model 4).

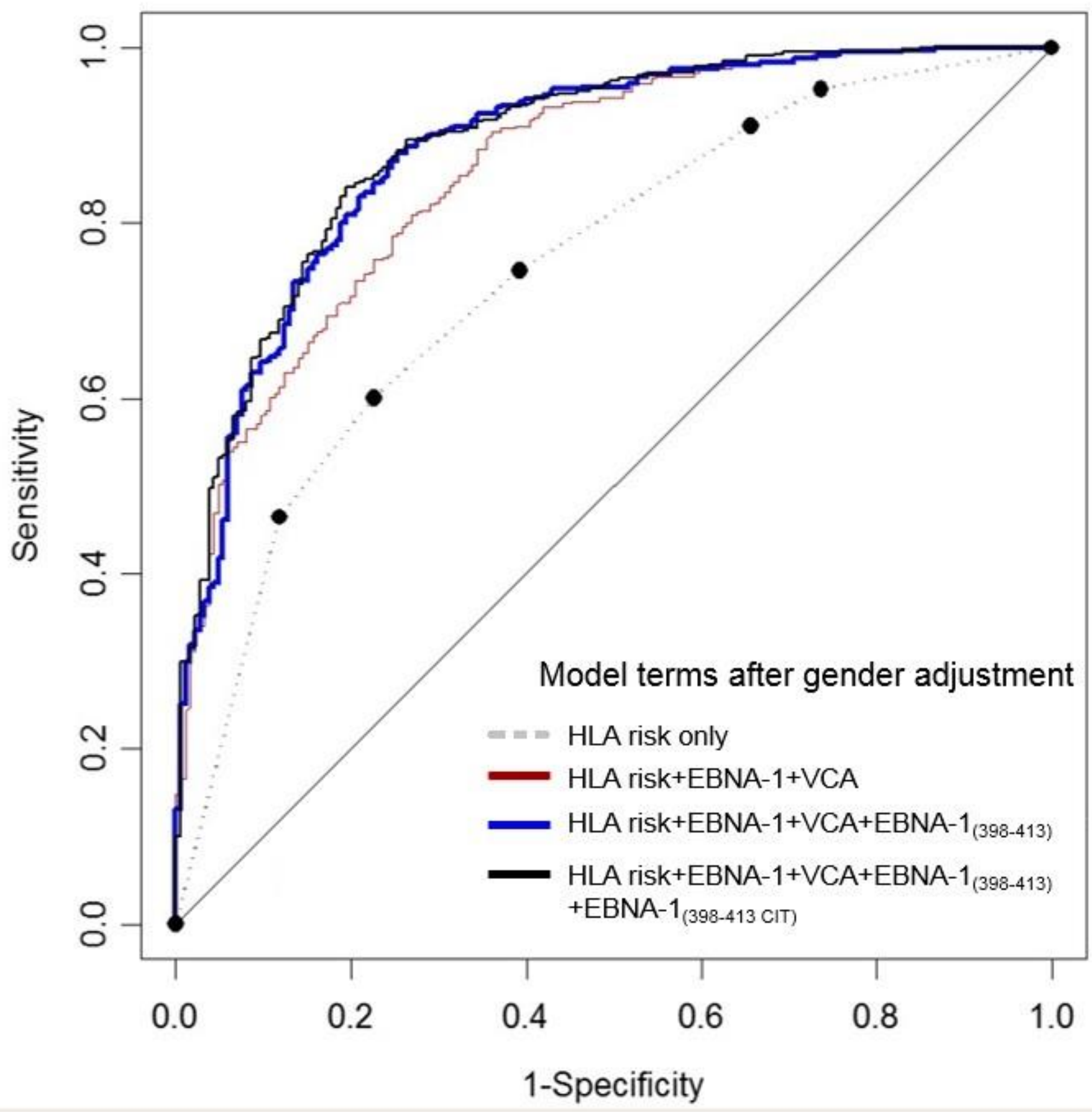


**Figure 5-6. Receiver operating characteristic (ROC) curve for logistic scores including HLA-DRB1 risk alleles, gender and ELISA values.** Receiver operating characteristic curves demonstrate the additional predictive power of including results from the commercial anti-EBNA-$1_{(long)}$, anti-VCA and additionally the in-house EBNA-$1_{(398\text{-}413)}$ and EBNA-$1_{(398\text{-}413\ CIT)}$ ELISA, compared to the HLA-DR risk groups alone.

## 5.3. Discussion

The recognition of citrullinated proteins by autoantibodies has been well-documented as a diagnostic utility in RA, and although some studies have reported citrullinated MBP as a target in MS (673, 674, 677, 678), there are currently no reports in regard to an antibody response to a citrullinated target, as utilized diagnostically in RA. This study is the first to review a citrullinated version of a latent EBV epitope as a target for immune response in MS. As described previously, serological studies on Australian MS cohorts have been limited over the past decades, in comparison to the research occurring across Europe and North America.

This study determined that serological measures of IgG antibodies specific for EBNA-1$_{(398\text{-}413\ CIT)}$ peptide were independently associated with MS risk, with seropositivity and antibody titres significantly increased in cases to controls. However, despite MS cases having higher mean antibody levels, there was no association with gender, HLA risk groups or age, as seen for uncitrullinated EBNA-1$_{(398\text{-}413)}$. Correlations of citrullinated and uncitrullinated EBNA-1$_{(398\text{-}413)}$ IgG levels (if not titrated to define an endpoint) suggest that both MS cases and healthy control individuals have antibodies against the citrullinated epitope at an equal or higher level than against the non-citrullinated form. This was surprising as the logged EBNA-1$_{(398\text{-}413\ CIT)}$ IgG levels were significantly higher in the MS cohort, and literature suggests an increased citrullinated response in MS. The citrullinated response may also reflect a pro-inflammatory environment of the individual. Additionally, in the logistic regression model, inclusion of the EBNA-1$_{(398\text{-}413\ CIT)}$ values reduced the significance of the EBNA-1$_{(398\text{-}413)}$ antibody levels in MS risk. If this immune response is independent of these factors, it may be reflective of a more pro-inflammatory environment in MS patients, rather than a specific targeted immune response, as seen in EBNA-1.

It has been identified that PAD is able to localize to the nucleus, making citrullination of the EBNA-1 protein possible (675). However, past studies focusing on immune responses to αß-C (700), found increased responses to this protein in MS cases. Sundqvist and colleagues (166) suggested from data by Steinmann *et al* (701) showed that MS patients have a strong T cell mediated autoimmunity towards αß-C, and that it could be possible that high anti-EBNA1 antibody levels might lead to increased autoimmunity against αβC, thereby increasing inflammation. A further question is whether the antibodies measured were specific for what could be a citrullinated version of EBNA-1, or a citrullinated version of the αβC protein, as a form of cross-reactivity, which could imply autoantibodies produced in the CNS later circulate in the periphery. Vossenaar *et al* (702) postulated that attributes of citrullinated proteins contribute to immune responses in multiple ways including increased PAD expression, more successful presentation of citrullinated protein by dendritic cells, plasma cells making antibodies against the citrullinated protein and the citrullinated protein-bound IgG antibodies potentially binding with higher affinity to $F_{c\Upsilon}$ receptors of macrophages.

Despite the less significant results for citrullinated-targeted antibody responses compared to the uncitrullinated form of EBNA-$1_{(398\text{-}413)}$, further research into citrullination is warranted. Potential studies could include modelling citrullinated forms of the epitopes to see if high-risk HLA alleles present the citrullinated forms more efficiently than the non-citrullinated epitopes, as seen in RA (650), measuring PAD enzyme expression in blood, brain and CSF, and identifying whether anti-EBNA-$1_{(398\text{-}413\ CIT)}$ antibodies increase or decrease over time in relation to disease progression. It has been shown that patients with more progressive MS forms have increased citrulline residues in the brain, and higher citrullination of MBP could occur by increased PAD2 synthesis, which could be affected by promoter methylation (674). Identifying these citrullinated targets and their mechanisms of control, could help elucidate whether

citrullination is a cause, by-product or result of MS pathogenesis, which may lead to more targeted therapeutic strategies.

## 5.4. Summary

Results from this study suggest that MS patients have higher antibody levels specific for EBNA-$1_{(398\text{-}413\ CIT)}$. A strong correlation was also seen between EBNA-$1_{(398\text{-}413)}$ and EBNA-$1_{(398\text{-}413\ CIT)}$. Targeting citrullinated forms of the epitope may only be seen in a subset of patients, and an elevated IgG antibody response to the citrullinated form is not seen unless there is an equally high EBNA-$1_{(398\text{-}413)}$ IgG antibody response. Including EBNA-$1_{(398\text{-}413\ CIT)}$ into a logistic regression is statistically significant, but when reviewed with other demographic and serological values, these values don't provide a significant improvement of the risk model, as seen in the ROC curve. The regression model suggests that the measurement of antibodies directed against citrullinated EBNA-1 does not have the same diagnostic values as seen in RA. However, citrullination is still an important process which occurs in MS and seems to be involved in the inflammatory pathway which needs further investigation.

# 6. Identification of IgG subclasses against EBNA-1$_{(398-413)}$

## 6.1. Introduction

### 6.1.1. Immunoglobulin gamma subclasses and their role in immunity

Sixty percent of total immunoglobulins in plasma are immunoglobulin gamma (IgG). These antibodies are most important in secondary antibody responses against infection, as part of immunologic memory, and the adaptive immune system. While IgM antibodies are the first to appear following viral infection (Figure 1-9), maturation of early IgM antibody responses is driven by repeated contact of B cells with a cognate antigen and cytokine stimulation of the immunoglobulin. This results in switching of the heavy chain, producing different antibody isotypes and subclasses (703), including IgG. IgG against EBV are detectable 3-6 weeks post-infection, and persists at relatively constant levels for life. Following antigen-binding, IgG has three major effector functions: activation of the complement cascade, opsonisation and fixation (Figure 6-1). Their main role is not to destroy foreign bodies or infected cells, but rather to 'tag' them for destruction by other immune cells. This communication is elicited by the pathogen-bound IgG antibodies binding to Fcɣ receptors, which are present on surfaces of macrophages, neutrophils and NK cells. The combination of antibody-mediated immunity show that IgG is often the first line in defence against an influx of antigen or infected cells, such as the lytic phase or 'reactivation' in which replication of EBV takes place.

IgG is composed of four subclasses (IgG1, 2, 3 and 4) which exist in different quantities in a healthy adult immune system (Table 6-1) dependent on the B cell environment (704) and differentiate by structure of the hinge region. Each subclass can be increased by stimulation from certain antigens, with IgG1 and IgG3 predominantly targeting presented viral antigens, IgG2 targeting mostly lipid polysaccharides while IgG4 has been suggested to have a role in tolerance to allergens and responses to certain infectious agents (705, 706). IgG binding of antigen often induces an effector cell response such as phagocytosis, degranulation, antibody-

dependent cell-mediated cytotoxicity (ADCC), inflammatory cytokine production and mediates activation of mast cells, monocytes and macrophages (707-709). This can in part be attributed to high and low affinity Fcɣ receptors present on immune cells which can bind to IgG subclasses with different strength (710). As IgG antibody subclasses differ in these abilities (Table 6-1), their biological functions are subclass-specific and can be associated with disease outcome (707, 711, 712).

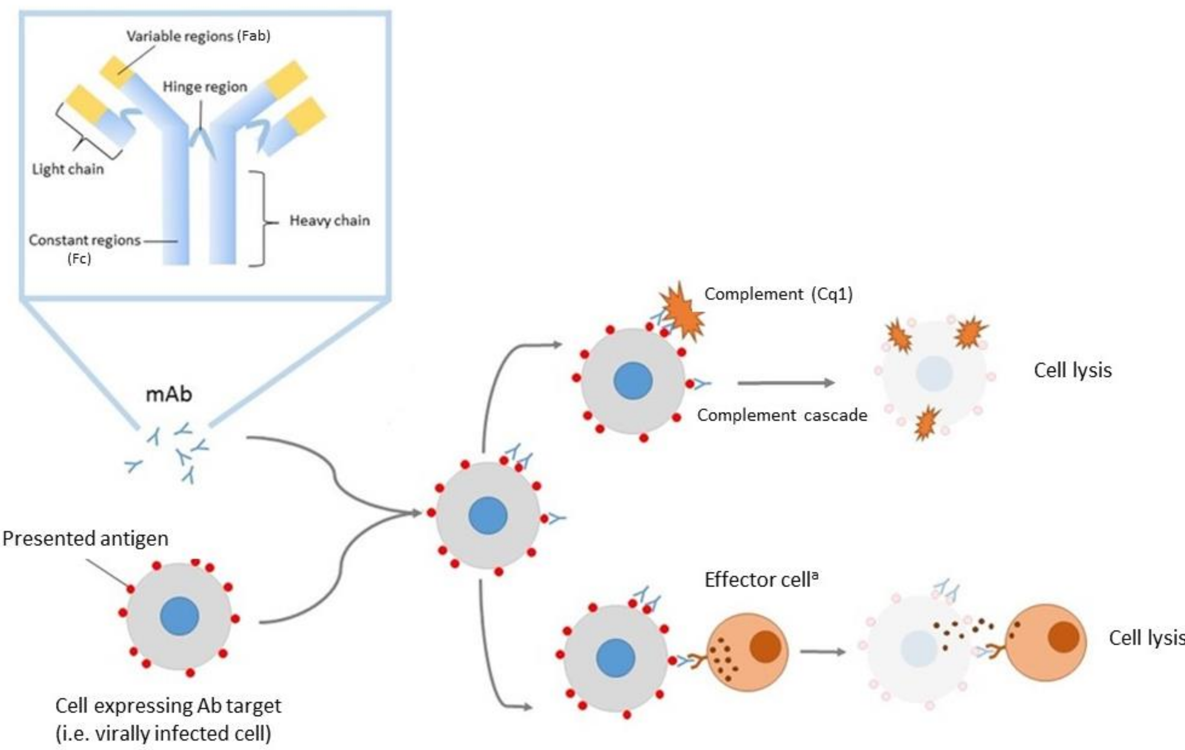


**Figure 6-1. The multi-functional effector role of IgG antibodies.** Adapted from (713). IgG antibodies act to identify foreign targets (antigens) and elicit an immune response through complement or by effector cells ([a]Natural killer cells/ neutrophils). mAb: monoclonal antibodies; meaning each antibody has the same target for the variable region (Fab).

**Table 6-1. Summary of Immunoglobulin gamma (IgG) subclass contribution to total IgG and comparative function.**

| | IgG1 | IgG2 | IgG3 | IgG4 |
|---|---|---|---|---|
| **Mean adult serum level (g/l)** | 6.98 | 3.8 | 0.51 | 0.56 |
| **Relative Abundance (%)** | 60 | 32 | 4 | 4 |
| **Complement activation** | ++ | + | +++ | - |
| **Opsonization** | +++ | +/- | ++ | + |
| **Phagocyte binding** | + | - | + | +/- |
| **Sensitization for killing by NK cells (ADCC)** | + | - | + | - |

Adapted from (714, 715). ADCC: Antibody-Dependent Cell-mediated Cytotoxicity.

### 6.1.2. EBV IgG subclass prevalence in MS

The presence of IgG antibodies within OCB in CSF is a hallmark of MS diagnosis, and IgG as well as complement have been found in MS lesions (542). The IgG OCB have been found to be reactive against EBNA-1 (478). The abnormal presence of antibodies and B cell clones in CSF and lesions, respectively, may suggest a pathogenic role in the disease (707). Past studies have investigated IgG subclasses of OCB, to help identify the microenvironment within the CNS. IgG1 and IgG3 have been reported as elevated in MS CSF (716, 717), and Grimaldi *et al.* (718) identified that six of ten MS patients with IgG1 OCBs showed additional reactivity against IgG3 or IgG4, suggesting a "microheterogeneous" composition. Greve *et al* (704) and Di Pauli *et al*. (707) reported higher IgG1 in CSF of MS patients compared to healthy controls and other neurological disorders, respectively. Neither study reported any difference in total IgG, IgG1, IgG2 or IgG3 in serum. However, both studies supported that elevated IgG1 and IgG3 reflect "type 1 immunity", a response caused by IFNɣ-induced antibody isotype switching for IgG subclasses (719). Additionally, Torkildsen *et al* reviewed that increased expression of

IgG-binding Fcɣ receptors has been identified in MS lesions (540) and reported Fcɣ receptor polymorphisms associated with MS, but this has not been confirmed in other studies so far (720-723).

One of the theories behind MS pathogenesis is molecular mimicry between viral (such as EBV) and self-peptides, and so levels of IgG subclasses specific for autoantigen or viral targets have also been investigated. Egg *et al.* (724) reported that MS relapse patients were more often anti-myelin oligodendrocyte glycoprotein (MOG) and anti-MBP IgG3 positive than those in remission, and identified reduced anti-MOG IgG3 titres in patients treated with intravenous immunoglobulin (Ig) or interferon-beta. They found IgG1>IgG2>IgG3 but no IgG4 against MOG, with IgG1 significantly associated with IgG3. The group did suggest that IgG2 responds, in a T cell independent manner, to carbohydrates, which could be targeted during myelin destruction, but levels would be higher in lesions than in circulation. Wakiguchi *et al* (725) found only elevated EBV-specific IgG1 in the serum of chronic EBV infection paediatric cases, whereas in MS adult cases, Lünemann *et al.* (480) reported non-significant differences for anti-EBV lysate IgG1 compared to healthy controls. However, Lünemann's group did identify elevated EBNA-1$_{(458\text{-}641)}$-specific IgG1 in MS CSF compared to controls, with no significance for IgG2 or IgG4. They did not detect any anti-EBNA-1 IgG3 in MS cases or controls. More recently, Cepok *et al.* (478) found that the oligoclonal IgG in MS CSF were cross-reactive for EBNA-1 protein, a potential molecular mimicry target. Obtaining CSF samples is difficult, but measuring circulating antibodies could contribute to understanding ongoing demyelination due to MS OCB in CSF being potentially attributed to a dysfunctional BBB (572). To date, anti-EBV subclass data has only been collected from small cohorts. The importance of IgG subclasses is that in addition to measuring antibody response against a viral target, subclass specificity can characterize the immune response.

To examine the IgG subclass responses in MS, the study described in this chapter used samples from the previously identified cohorts of MS patients and healthy controls (Busselton Study). Given the available samples, the following questions were addressed in this chapter:

1. Are there differences in antibody response, positivity and titres, against EBNA-1$_{(398\text{-}413)}$ specific IgG1, IgG2 and/or IgG3 subclasses between the two cohorts?
2. Are results influences by gender, age or HLA risk status?
3. What is the correlation of subclass antibodies and EBNA-1$_{(398\text{-}413)}$ $IgG_{Total}$ from Chapter 4?
4. Could these factors contribute to a logistic regression risk model to determine MS risk?

## 6.2. Results

### 6.2.1. Anti-EBNA-1$_{(398-413)}$ IgG1, IgG2 and IgG3 in cases and controls

In contrast to previous studies measuring mainly IgG subclasses against entire EBV proteins, we focused with our analysis on IgG subclasses against a very small section of EBNA-1. A surprisingly small proportion of samples had detectable anti-EBNA-1$_{(398-413)}$ IgG subclass levels by our in-house ELISAs (Figure 6-2), with the highest positive proportion being IgG1 (42% for MS, 28% controls; $p<0.01$). IgG2 was only positive in very few samples from either cohort, but significantly more samples were above the lower cut off in the MS group (8% MS, 3% controls; $p<0.05$) and no significant differences were observed for IgG3 positivity between cohorts (3% MS, 5% controls, $p>0.05$). Only 25 of the 426 MS patients were positive for more than one subclass against EBNA-1$_{(398-413)}$, with the majority of those positive for IgG1+2 (n=19), next to IgG1+IgG3 (n=2), IgG2+IgG3 (n=1) and IgG1+IgG2+IgG3 (n=2). Although numbers were small, MS samples had a significantly higher mean titre for IgG1 ($p<0.05$) and IgG3 ($p<0.05$), but not for IgG2 ($p=0.3$). Furthermore, there was no detectable influence of HLA-DR profiles, gender or age on subclass epitope-specific EBNA-1 antibody levels in controls or MS samples ($p>0.3$). After adjusting for MS/control effects, $\log_{10}$ ($IgG_{Total}$ levels) were positively associated with IgG1 ($p=0.0002$), IgG2 ($p=7.8 \times 10^{-16}$) and IgG3 ($p=0.035$). When considered jointly, IgG3 lost significance after adjusting for IgG1 and IgG2 ($p=0.2$), while IgG1 ($p=0.01$) and IgG2 ($p=1.3 \times 10^{-14}$) were independently associated with $IgG_{Total}$. Therefore, IgG2 appears to have the dominant association, while IgG1 is still strongly significant. Inter-assay variability of serially diluted IC for this assay was 16.8-21.5%.

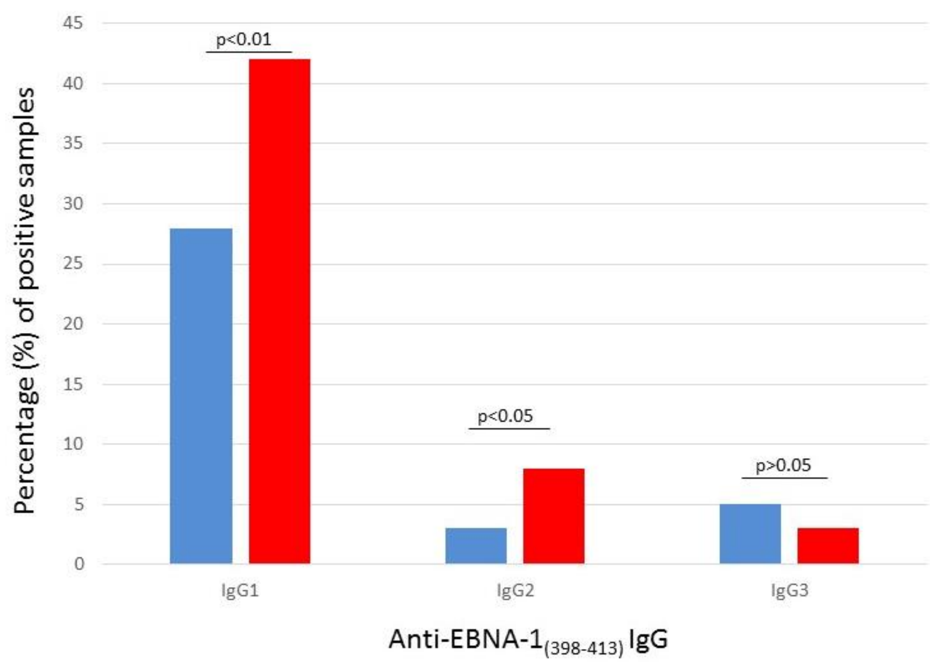


**Figure 6-2. Seropositivity of anti-EBNA-1$_{(398-413)}$ IgG subclasses in MS and control cohorts.** Percentage of positive samples between MS (n=427; red) and controls (n=186; blue) for EBNA-1$_{(398-413)}$ IgG subclass ELISAs. MS had significantly more positive samples for both IgG1 and IgG2.

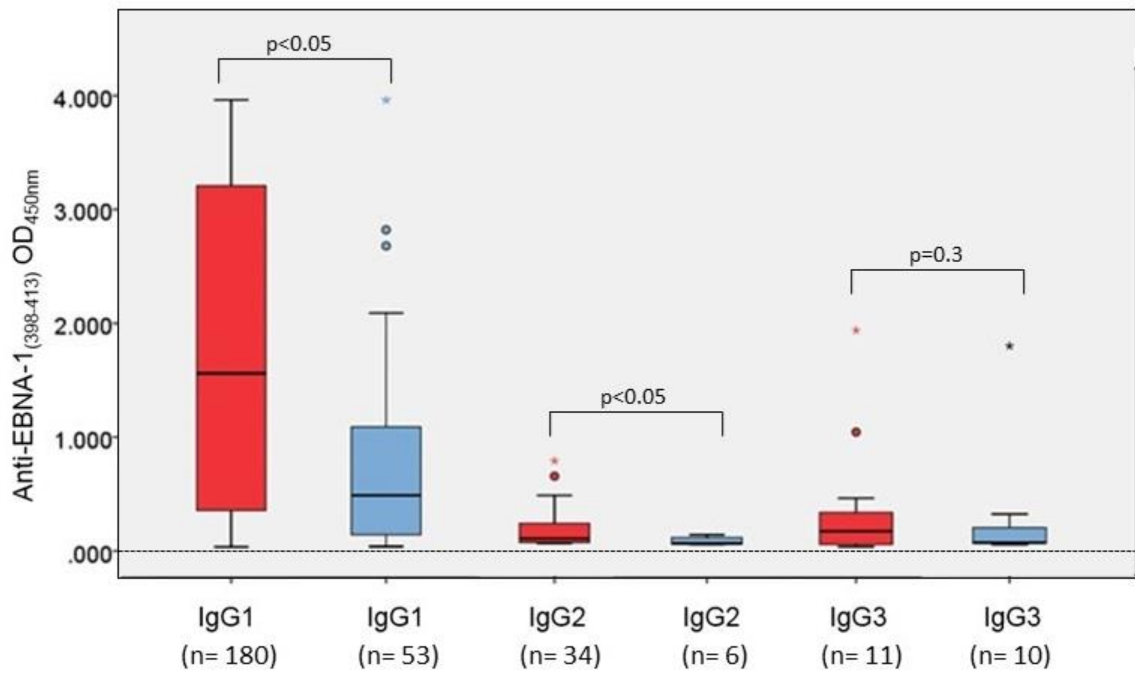


**Figure 6-3. ELISA results for both cohorts split by IgG subclass.** Serum anti-EBNA-$1_{(398\text{-}413)}$ IgG1, IgG2 and IgG3 $OD_{450nm}$ from MS (red) and healthy controls (HC; blue). MS is significantly higher than controls for IgG1 and IgG2 (**$p<0.05$), but no significant difference was seen for IgG3.

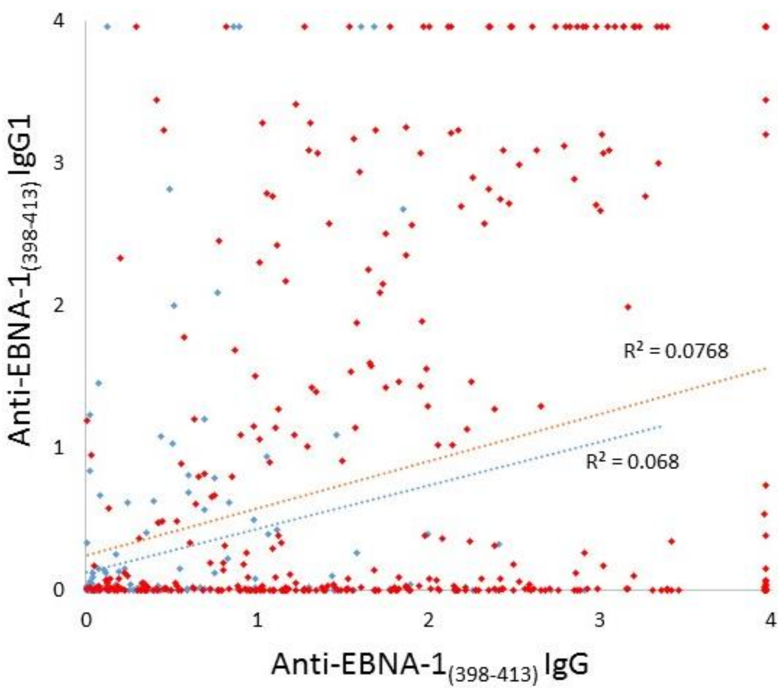


**Figure 6-4. Correlation of anti-EBNA-1(398-413) total IgG and EBNA-1(398-413) IgG1.** MS (red) had consistently higher IgG1 values than healthy controls (blue). Samples can only reach an optical density (OD) of 4.0.

### 6.2.2. Logistic regression and receiver-operating characteristic curve analyses

Adjusting for risk factors (HLA, previous commercial and in-house ELISA data) and gender, anti-EBNA-$1_{(398\text{-}413)}$ IgG1 (p=0.005), IgG2 (0.027) and IgG3 (p=0.029) were independently significant risk factors. IgG1 and IgG2 were positively associated with MS cases while IgG3 was negatively associated. As demonstrated in Table 6-2 (Model 5), inclusion of EBNA-1, VCA and EBNA-$1_{(398\text{-}413)}$ resulted in abrogated IgG1 and IgG2 effects (p=0.8, p=0.53 respectively), while IgG3 remained significant (p=0.008) for reducing risk. Inclusion of the IgG subclass values did not abrogate effects of VCA, EBNA-1 or EBNA-$1_{(398\text{-}413)}$. Similar results were seen when results for EBNA-$1_{(398\text{-}413)}$ were omitted, with EBNA-1 and VCA values abrogating IgG1 and IgG2. This model shows significance for protective (low risk) HLA-DR alleles (OR 0.39, p=0.0043) but there was no significant association for high risk HLA-DR alleles (p=0.31), similar to the inclusion of EBV-specific ELISA results described in Chapter 4. Despite significance of IgG3, addition of the assay results show minimal contribution to improving the logistic regression, as evident from the ROC curves shown in Figure 6-5 based on the linear-logistic model scores. With the addition of values for anti-EBNA-$1_{(398\text{-}413)}$ IgG subclasses, the final model was defined by $1.90 - 0.942(HLA\text{-}DR_{low\text{-}risk}) + 0.34(HLA\text{-}DR(_{high\text{-}risk}) - 0.96(Male) + 1.54 \times logVCA + 1.25 \times logEBNA_{(long)} + 1.24 \times logEBNA_{(398\text{-}413)} - 0.05 \times logEBNA_{(IgG1)} - 0.58 \times logEBNA_{(IgG2)} - 0.1.83 \times logEBNA_{(IgG3)}$. This model provided a sensitivity of 391/426=92% and specificity of 121/186=65% at a cut-off logistic value of ≥0 (OR 20.8). However, this, in fact, reduces the discrimination capability in model 3, previously described in Chapter 4.

**Table 6-2. Analysis of genetic and serological MS risk factors using progressive logistic regressions.**

| | Model 2 | | Model 3 | | Model 5 | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** | **Odds Ratio** | **P value** |
| **HLA-DR High risk group** | 1.40 | 0.26 | 1.26 | 0.47 | 1.39 | 0.31 |
| **HLA-DR Low risk group** | 0.42 | 0.0061 | 0.38 | 0.003 | 0.39 | 0.0043 |
| **Gender (Female)** | 2.63 | $3.5\times10^{-5}$ | 2.73 | $4.0\times10^{-5}$ | 2.62 | $9.5\times10^{-5}$ |
| **EBNA-1$_{(long)}$ OD (log)** | 6.76 | $1.8\times10^{-14}$ | 3.17 | $3.0\times10^{-5}$ | 3.49 | $1.2\times10^{-5}$ |
| **VCA OD values (log)** | 4.96 | $6.3\times10^{-7}$ | 4.30 | $2.3\times10^{-5}$ | 4.69 | $1.2\times10^{-5}$ |
| **EBNA-1$_{(398\text{-}413)}$ OD (log)** | | | 3.47 | $1.7\times10^{-9}$ | 3.46 | $4.8\times10^{-9}$ |
| **EBNA-1$_{(398\text{-}413)}$ IgG1 OD (log)** | | | | | 0.95 | 0.79 |
| **EBNA-1$_{(398\text{-}413)}$ IgG2 OD (log)** | | | | | 0.55 | 0.53 |
| **EBNA-1$_{(398\text{-}413)}$ IgG3 OD (log)** | | | | | 0.16 | 0.0078 |

Logistic regressions for significance of MS risk factors identified previously by this group including commercial ELISA values (Model 2), EBNA-1$_{(398\text{-}413)}$ results (Model 3) and EBNA-1$_{(398\text{-}413)}$-specific IgG subclasses (Model 5). EBNA-1$_{(398\text{-}413)}$-specific IgG1 and IgG2 did not reach significance in this model when other ELISA values were included.

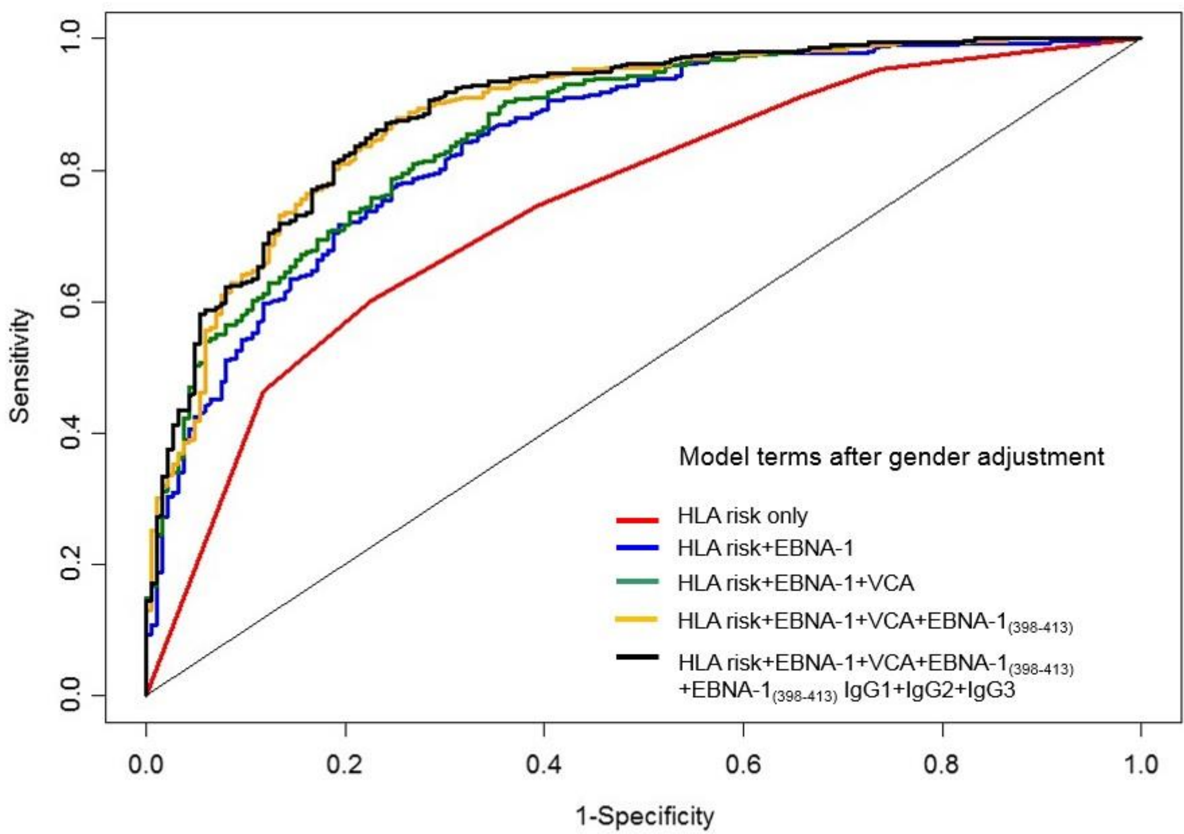


**Figure 6-5. Receiver operating characteristic curve for logistic scores including HLA-DRB1 risk alleles, gender and ELISA values.** ROC curves demonstrate the additional predictive power of including results from the commercial anti-EBNA-1$_{(long)}$, anti-VCA and additionally the in-house EBNA-1$_{(398\text{-}413)}$ and EBNA-1$_{(398\text{-}413)}$ subclass ELISA, compared to the HLA-DR risk groups alone.

## 6.3. Discussion

Defining levels of total and antigen-specific IgG subclasses is important due to their different affinities for antigen targets and biological functions. This chapter reviewed an in-house assay to quantitate the amount of EBNA-1$_{(398-413)}$-specific antibodies within the subclasses of IgG1, IgG2 and IgG3 using the automated ELISA protocol from Chapters 3 and 4. It was originally expected that anti-EBNA-1$_{(398-413)}$ IgG1 would be the most prevalent in all samples positive for IgG$_{Total}$ against this epitope, but this was not seen. A majority of patients and controls had non-detectable IgG subclass levels, which was unexpected considering almost all individuals were positive for EBNA-1$_{(398-413)}$ IgG$_{Total}$. It was also surprising that more than one subclass was only detected in a minority of "positive" samples. It is possible that the secondary IgG subclass antibodies, which were purchases from a different company, were less sensitive than the total IgG secondary antibody, which would mean more individuals were positive for anti-EBNA-1$_{(398-413)}$ subclasses, but were below the detection limit of the assay. On the other hand, it cannot be excluded that quantitative differences in detection occurred due to different chemical binding specificities of the IgG2 antibodies, such as IgG$_{Total}$ and IgG2 targeted the Fc portion of patient antibodies, while IgG1 and IgG3 targeted the hinge region (Figure 6-1).

To date, the only MS-associated EBV-specific subclass research that has been reported is from Lünemann *et al.* (480) who measured all four subclasses against EBNA-1 protein excluding the glycine-alanine region. His group found significantly higher anti-EBNA-1 IgG1 in MS patients compared to controls, and detected no IgG3 in MS or control cohorts and also detected anti-EBNA-1 IgG4 in a subset of patients. Our own study did not measure IgG4 due the lack of evidence in the literature that would indicate diagnostic or pathogenic role for IgG4 antibodies in MS. Nonetheless, our study did find significant differences in IgG3 levels between cohorts, which was not identified in the Lünemann study (480), despite their EBNA-1 protein being a

larger target and identifying polyclonal antibodies compared to our short EBNA-$1_{(398\text{-}413)}$ peptide. However, Lünemann's cohorts were small- with only 20 MS patients and 16 EBV-positive controls. We could show for the first time that IgG3 against EBNA-$1_{(398\text{-}413)}$ is detectable, but may require larger cohorts as less than 10% of either group in our study had detectable anti-EBNA-$1_{(398\text{-}413)}$ IgG3 levels. This conclusion could also be drawn regarding significant differences in positivity but not titre for anti-EBNA-$_{1(398\text{-}413)}$ IgG2 in this study. Our study supports Lünemann's findings of elevated IgG1 against EBNA-1, with significantly higher titres and more IgG1-positive individuals in the MS cohort. This is important because IgG1 significance is maintained despite using a much smaller portion of EBNA-1 as a target. Overall, IgG1 elevation could indicate a 'type 1 immunity' response in MS patients. The most interesting finding from this study was that IgG3 elevated levels were detected more often in healthy controls, and indeed proved statistically significant in reducing MS predisposition in our risk model. This is surprising as IgG3 and IgG1 were both previously associated with the "type 1 immunity" (704). Vidarsson *et al.* reviewed that IgG3 and IgG1 are both expected in response to viral infections, and IgG3 is expected first in the course of infection (714). It may be possible that IgG3 reflects early detection and control of EBV, while IgG1 is elevated in individuals with sustained EBV reactivation.

Similar to the total IgG against EBNA-$1_{(398\text{-}413)}$ in chapter 4, none of the subclass IgG data was significantly associated with HLA risk status, gender or age. This would support the earlier conclusions that the peptide is a true B-cell-epitope, but considering the small number of positive samples, a control cohort should be tested to validate this finding. IgG1 and IgG2 were independently associated with $IgG_{Total}$, but it should again be noted that these associations are made with quite small sample sizes for the IgG subclass data. In a risk model, each subclass was independently significant as a risk factor, and interestingly IgG3 had a negative association

with MS risk. When commercial and $IgG_{Total}$ against EBNA-$1_{(398-413)}$ values were included, only IgG3 remained significant, and subclass data had no effect on the other ELISA findings. Addition of IgG subclasses in the model resulted in the logistic regression reducing discrimination capability. Therefore, these results do not support significant benefit from including anti-EBNA-$_{1(398-413)}$ IgG subclass ELISAs in a validation cohort due to the large proportion of samples with no detectable levels, and instead focus on the functional properties of these antibodies would appear more beneficial. Additionally, for future research it would be interesting to investigate Fcɣ receptor expression in this cohort to compare with subclass data, despite conflicting literature, as identifying a functional link between IgG antibodies and Fcɣ receptor expressing cells in MS CNS would be beneficial to understanding the significance of these elevated anti-EBNA-1 antibodies in MS. Lastly, the large cohort could be utilized to study as Wuhrer *et al*. (726) suggested glycosylation of IgG subclass changes are associated with inducing antigen-specific immune responses (727).

## 6.4. Summary

Results from this study suggest that MS patients have higher IgG1 and IgG2 levels specific for EBNA-$1_{(398-413)}$, however a majority of samples were seronegative for each IgG subclass tested. IgG1, IgG2 and IgG3 were positively and independently associated with $IgG_{Total}$ against EBNA-$1_{(398-413)}$, but when combined, only IgG1 and IgG2 remained significant. No association could be observed for any of the subclasses with HLA risk alleles, gender or age. Including these factors into a logistic regression model showed that IgG1 and IgG2 were not significant, but IgG3 was statistically significant, contributing a lower risk for MS, yet not improving the risk model, as seen in the ROC curve. Our results presented in this chapter suggest a mainly Th1-mediated immune response against the EBNA-$1_{(398-413)}$ antigen.

# 7. Whole serum and anti-EBNA-1$_{(398-413)}$ IgG cross-reactivity with brain antigens tested by protein macroarray, and *in vitro* effect of MS serum on selected human-derived cell lines.

## 7.1. Introduction

### 7.1.1. Cross reactivity of EBV-specific antibodies with autoantigens

EBV is known to share homology and functions with a range of protein sequences in humans, which is a vital contribution to the virus' ability to evade the immune system. EBV remains latent within B cells over decades of an individual's life, however phases of lytic replication enable the host to detect and target the virus. Studies have identified that in MS, EBV-specific immune cells are cross-reactive with myelin antigens (480, 491, 492), and Elliott *et al.* (728) identified MS patients with antibodies reactive against myelin. However, anti-MBP (729) and anti-MOG (730) antibodies are also common in people with a healthy immune system so just the presence of autoantibodies does not seem sufficient to cause disease. One of the prominent theories behind MS pathology is the trigger of immune T cell cross-reactivity, where immune cells specific for EBV erroneously recognize self peptides as foreign and mounting a response (483). Further to the implications of cross-reactive T cells, anti-EBNA-1 antibodies have also been reported in MS CSF (476, 478, 572). Our short targeted EBNA-$1_{(398\text{-}413)}$ peptide shares amino acid sequence homology with αßC, which raises the question whether the antibodies against this region themselves, like the EBV-specific T cells, can cross react and target myelin or the αßC component (480). Testing for such antibody cross-reactivity in autopsied tissue would be most relevant, but limited access to human samples makes this difficult. There has been considerable research utilizing mouse models, but many difficulties are needed to be overcome translation of findings (731). *In vitro* models do have some limitations but are a good alternative of investigating cellular and molecular mechanisms of disease pathogenesis. The hexSelect macroarray was chosen as it consists of a variety of expressed recombinant human brain proteins. It can serve as a very useful method for detection of autoantibodies utilised as part of a screening process to identify proteins of interest for further experimentation.

### 7.1.2. Use of hexSelect array for MS and other autoimmune diseases

The hexSelect array (Source BioScience, USA) has been used in multiple publications, including cardiac disease (732), colorectal cancer (733) and mRNA processing bodies in autoimmune disease (734). The array can help identify cross-reactivity of antibodies to 37,000 expressed recombinant proteins (including a large spectrum of brain proteins), spotted in duplicate onto a 30cm x 30cm membrane. The array can help in screening for new antibody targets, identifying disease autoantibody signatures and could be used to understand disease pathogenesis and potential targets for therapeutic strategies. Cepok *et al.* (478) used the array to identify antibody reactivity of 12 MS CSF and 5 control samples, and identified and confirmed 0-10 "expression clones" specifically stained above background in each patient. Strongest CSF reactivity against two peptides: EBNA1 (302-641) and BRRF2, which are both derived from EBV proteins, was found in 3 patients. However, the arrays used by Cepok's group were blotted on two separate membranes and proteins codes were different from the hexSelect array used in our study. Understanding the potential-cross reactivity of antibodies may assist in understanding disease pathogenesis, help with diagnosis and aid in developing new treatments.

### 7.1.3. In vivo and in vitro models for MS pathogenesis

There are currently multiple animal models for MS available, mainly used to test safety and efficacy of drug treatments, and pathological changes including citrullination as outlined in Table 5-1. Experimental Autoimmune Encephalomyelitis (EAE) animal models (including mice, guinea pigs, rats and primates) induce monophasic CNS damage following immunization with myelin antigens and therefore only reflect part of the underlying MS mechanism. These models can contribute to the understanding of the diseases pathogenesis and limitations in their application have been reviewed in detail before (735-738), including the difficulty of translating

treatments into clinical trials (739). From a cellular point of view, the mature CNS is a complex matrix of multiple different cell types (740). As the pathogenesis of MS is not fully understood, determining a cellular model including all relevant cell types for the disease is difficult. A range of different cell types have been shown to be involved in disease pathogenesis including: neuronal, glial, astrocytes and oligodendrocytes and combinations thereof have all been reviewed in MS studies (741). Multiple groups have worked towards *in vitro* models for MS, with focus on the BBB endothelium (742) and it's disruption by EBV infection (606). Others have focused on serum and antibody effects in cell cultures and their influence on binding abilities and degradation of immunogenic targets. Lily *et al.* (743) reported no significant difference in surface binding of MS autoantibodies to oligodendrocyte and neuronal cell lines compared with controls, but when stratifying by RRMS and SPMS they found higher binding of serum antibodies from RRMS compared to SPMS patients. Elliot *et al.* (728) added MS-derived immunoglobulin to rat spinal cord-derived astrocyte cell culture, reporting complement dependent demyelination in 30% of MS patients, with mediated axonal loss with two MS patient sera. The neuronal cell line SH-SY5Y has not been utilized in MS studies as it is obtained from the peripheral nervous system rather than CNS, and is therefore a good control cell line. In order to test out potential autoantibodies functionally, we chose two cell lines for our *in vitro* experiments. The oligodendrocytic cell line MO3.13, which has been used to test cell-penetrating peptides for the potential of re-establishing myelination (744) as well as identifying anti-MOG antibodies on demyelination (745). The neuronal cell line SH-SY5Y on the other hand was chosen as a control cell line. The cell line is obtained from the peripheral nervous system rather than CNS, and is therefore an ideal candidate to measure target effects. Both cell lines can also be differentiated further when stimulated with chemicals. This gives us the unique possibility to measure both effect to immature cells and to cells more accurately representing the mature cell type *in vivo* (744, 746).

The study described in this chapter used a subset of diagnosed MS patients from the PDDD-b and a control group from the Red Cross (RC) cohort. All samples had been tested on commercial and in-house ELISAs. The following questions were addressed in this chapter:

1. Does healthy control plasma contain IgG antibodies which target brain proteins?
2. Does MS serum have IgG antibodies which target brain proteins, and do these targets differ from healthy controls and between clinical courses for MS groups?
3. Can isolated anti-EBNA-1$_{(398-413)}$ IgG antibodies bind and target brain proteins, and if so are the proteins the same as from whole serum reactivity as expected?
4. What is the possible role of identified brain proteins targeted in MS in particular with regards to inflammation, and do they have common roles or co-localization in human tissue?
5. What is the functional effect of MS serum on brain derived cell culture in particular on viability?

## 7.2. Results

### 7.2.1. Study Cohort

For screening of potential autoantibodies, samples were pooled. The groups used in the macroarray were chosen from clinical information for MS and HLA-DRB alleles for Red Cross controls (Table 2-1). There was a 2:1 male to female ratio for the MS acute/relapse patients. Additionally, all SPMS patients were female, and the single PPMS patient was male. Red Cross controls (7 female, 3 male) were chosen for either having high risk, moderate or low risk/ "protective" HLA-DRB1 alleles. All three CIS patients had HLA-DRB1*1501, and one patient was homozygous.

### 7.2.2. Protein array binding whole serum and anti-EBNA-1$_{(398-413)}$ isolated IgG

As expected, whole serum had a higher background on the array than isolated IgG. One difficulty encountered in the experiment was that isolated antibodies which showed initially positive results (duplicate dots for one protein), tended to become negative due to fading within 10 minutes of ECL reagent addition. This finding raised questions regarding the durability and avidity of the isolated antibodies, as control dot blots performed with pooled MS serum did not fade as quickly as the macroarrays, using the same secondary antibody and ECL reagents (Figure S-4). However, several CIS MS antibodies were isolated, and when tested on ELISA were above the negative cut-off value. For photos of the protein arrays, positive spots above the background level were identified, and matched to the manufacturer's list of dotted proteins identified by their (X,Y) locations. Dots which were not identified in duplicate were not included, as per the manufacturer's instructions. Confirmed proteins (detected in duplicate) for each sample group are summarized in Table 7-1, with images of duplicates shown in Figure 7-1.

**Table 7-1. Proteins identified as reactive with MS and healthy serum IgG.**

| Protein Code | Protein Name | Acute MS serum | Acute MS antibody only | Progressive MS serum | HC plasma | HC plasma [2] |
|---|---|---|---|---|---|---|
| STMN4 | Stathmin-4 | 4 | | | 1 | 3 |
| HDAC5 | Histone deacetylase 5 | 1 | | | 1 | 2 |
| MBD3 | Methyl-CpG binding domain protein 3 | 1 | | | | |
| PIM3 | Serine/threonin-protein kinase Pim-3 | 1 | | | | |
| EPN1 | Epsin-1 | 1 | | | | |
| JMJD8 | Jumonji domain-containing protein 8 | 1 | | | | |
| SHB2 | SH2 domain-containing adapter protein B | 1 | | | | |
| HBA2 | Haemoglobin subunit alpha | | 1 | | | |
| EEF1A2 | Elongation factor 1-alpha 2 (Statin S1) | | 1 | | | |
| TROVE2 | 60 kDa SS-A/Ro ribonucleoprotein | | | 2 | | |
| NFKBIL2 | NF-kappa B inhibitor like protein 2 | | | 1 | | |
| KAT2A | Histone acetyltransferase | | | | 1 | |
| CENPB | Major centromere autoantigen B | | | | 1 | 2 |
| AMPD2 | AMP deaminase 2 | | | | 1 | |
| MAP1LC3A | Microtubule-associated protein 1A/1B light chain 3A precursor | | | | | 3 |
| UFC1 | Ubiquitin-fold modifier-conjugating enzyme 1 | | | | | 1 |
| AZGP1 | Zinc-alpha-2-glycoprotein | | | | | 1 |

Healthy control plasma (2) was tested after reusing the stripped membrane. Numbers indicate the number of duplicates identified across the whole membrane.

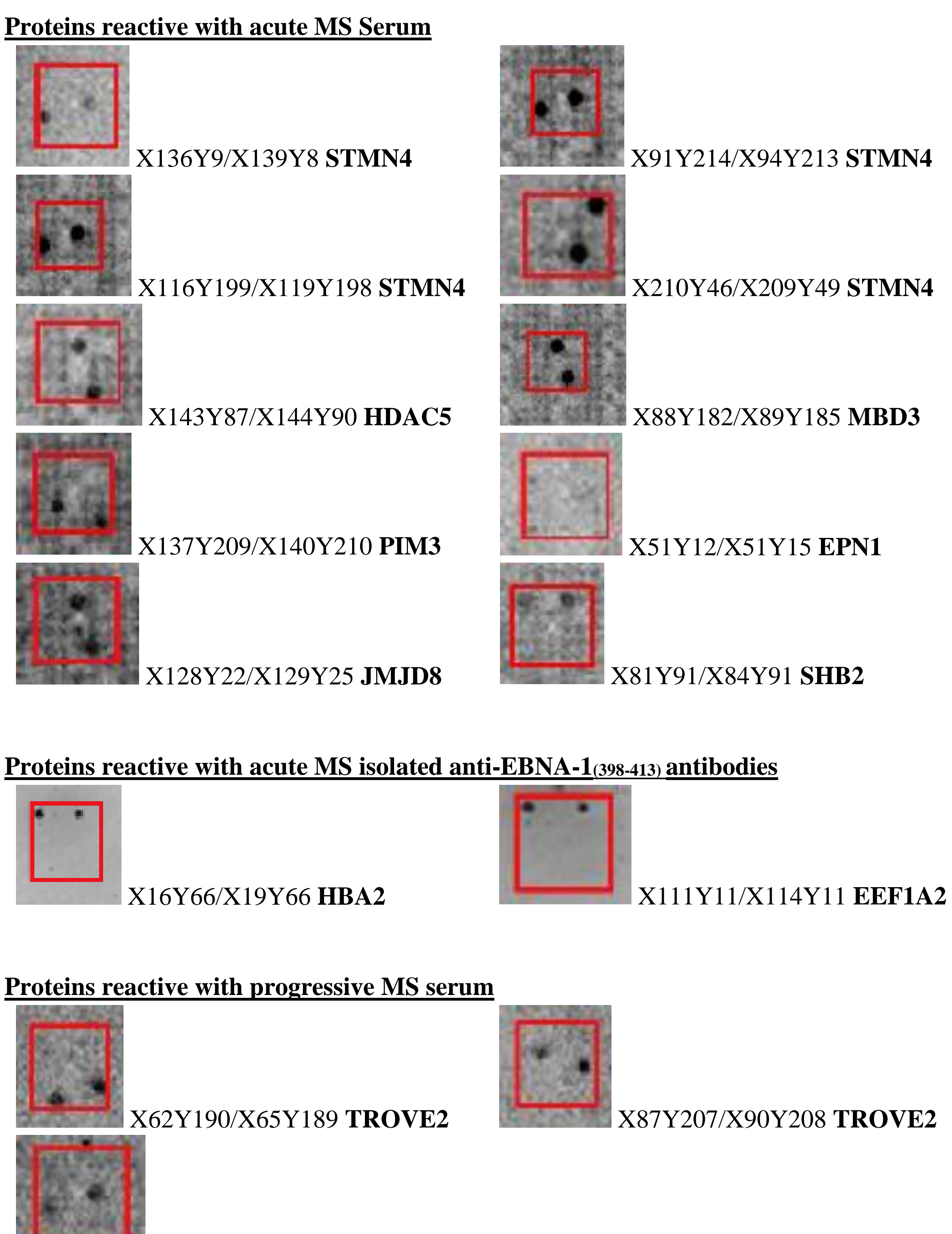


**Figure 7-1. Raw images of duplicates tested with the macroarray for each sample group.** Full names of protein codes are in Table 7-1 and Supplementary Table S-4.

**Proteins reactive with healthy plasma**

X97Y35/ X100Y34 **KAT2A**

X210Y46/X209Y49 **STMN4**

X96Y124/X99Y123 **AMPD2**

X197Y88/X198Y87/X198Y90/X199Y90 SOBP/**HDAC5**/ECSIT/**HDAC5**

**Proteins reactive with Healthy plasma [2] [post stripping membrane]**

X210Y46/X209Y49 **STMN4**

X136Y9/X139Y8 **STMN4**

X91Y214/X94Y213 **STMN4**

X143Y87/X144Y90 **HDAC5**

X198Y87/X199Y90 **HDAC5**

X67Y175/X70/Y174 **CENPB**

X1Y222/X1Y225 **CENPB**

X33Y72/X34Y75 **MAP1LC3A**

X202Y196/X202Y198 **MAP1LC3A**

X96Y204/X99Y203 **MAP1LC3A**

X57Y214/X60Y215 **UFC1**

X231Y231/X234Y231 **AZGP1**

**Figure 7-1. Raw images of duplicates tested with the macroarray for each sample group.** Full names of protein codes are in Table 7-1 and Supplementary Table S-4.

### 7.2.3. Cell culture

The cell lines, SH-SY5Y (neuronal) and MO3.13 (oligodendrocytic), were tested for mycoplasma contamination before experiments were conducted and both cell lines were found to be negative using the PCR technique (Figure 7-2). They were grown in flasks until 70% confluent (Figure 7-3) and kept between passages 7-14 for MO3.13 and 14-33 for SY5Y. The cell line MO3.13 was tested for its ability to differentiate using phorbol myristate acetate (PMA). Differentiation was successful as determined by morphological changes like elongation of oligodendrocytic arms (Figure 7-2C) when compared to undifferentiated length of cells (Figure 7-2B).

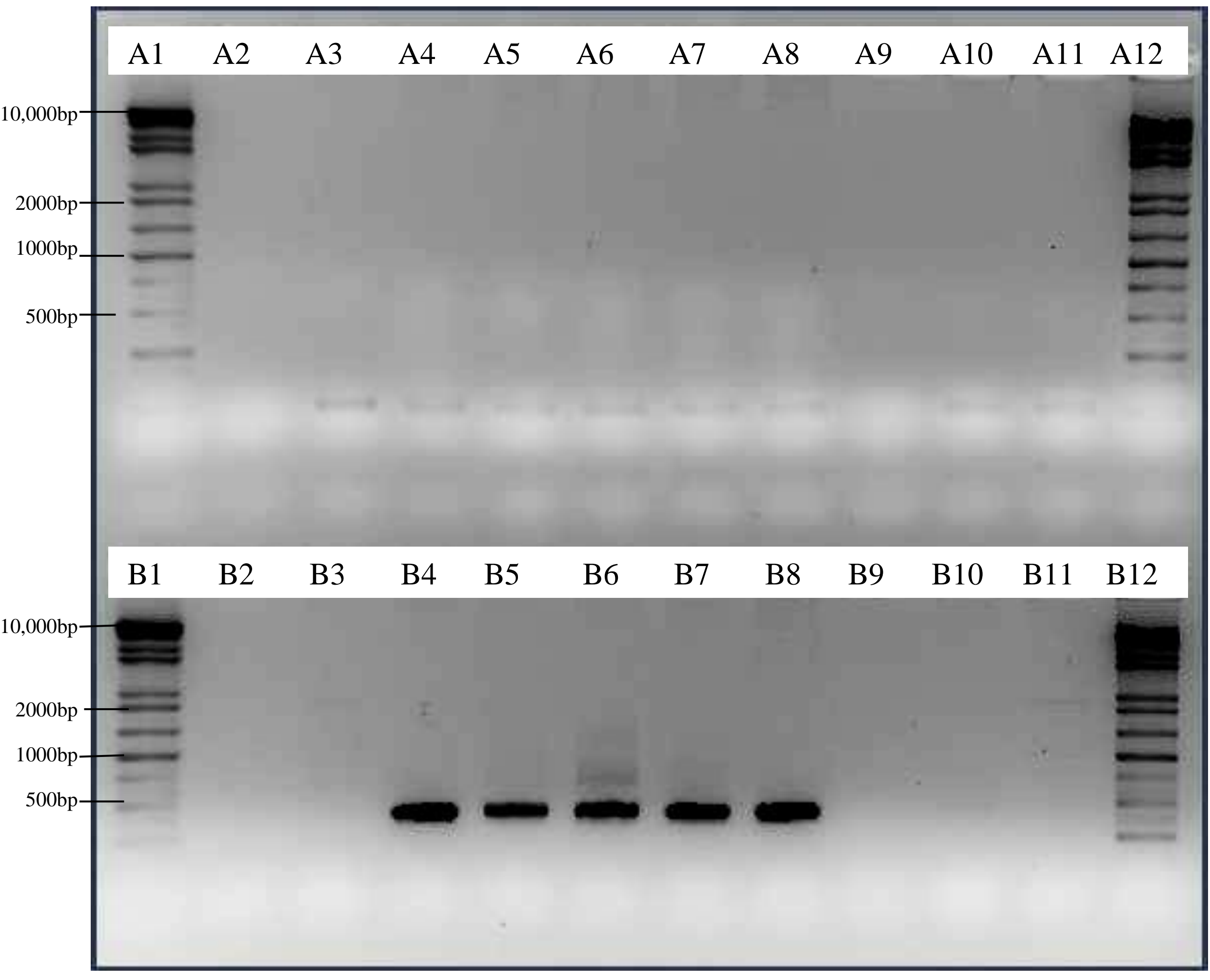


**Figure 7-2. Gel electrophoresis of Mycoplasma PCR testing of cell lines.** Row A is products from the positive HGH control, row B was products using the Leedman Mycoplasma PCR. 1kb ladder (1, 12), negative control (2), uninfected cell control (3), and positive *Mycoplasma* infected cell controls (B4-B8: *M. arginine; M. hominis; M. hyorhinis; M. fermentans; M. pharyngis*). DNA extraction negative control (B9) shows MO3.13 (B10) and SY5Y (B11) cell lines are negative for Mycoplasma contamination using IIID's Leedman Mycoplasma test.

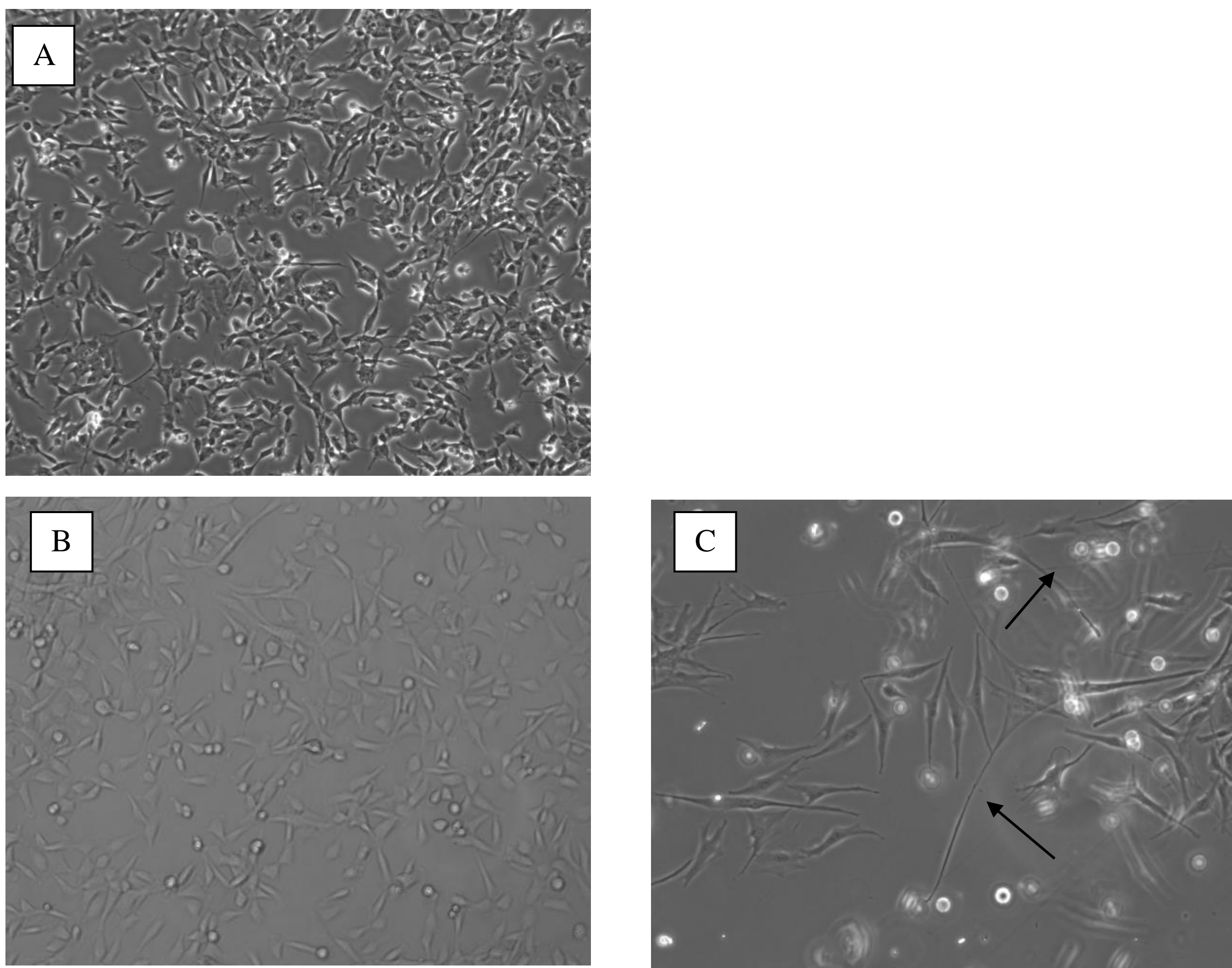


**Figure 7-3. Cell lines SY5Y undifferentiated (A), MO3.13 undifferentiated (B) and differentiated (C).** The black arrows identify the elongated arms of MO3.13. Magnification (40X).

### 7.2.4. MS serum effect on cell lines

Cell lines were tested in triplicate wells for viability after incubation with MS serum for 6 hours, 24 hours and 48 hours at two different concentrations by diluting 1:100 and 1:1000. A summary of averaged viability counts is shown in Table 7-2 (raw data in Supplementary Table S-5), and displayed in Figure 7-4. Logistic regression was used to identify any significant changes over time with and without serum due to relatively low number of cell counts. For oligodendrocytic MO3.13, there was a significant effect of time on viability ($p=0.005$), due to decreased viability at 48 hours, but no significance of either serum addition ($p=0.21$). For neuronal SHY-5Y, serum 1/1000 had a very different viability trend over time compared to no serum and serum 1/100 ($p=6.6\times10^{-6}$). For this, serum 1/1000 had a lower viability at 6 hours which increased by 48 hours, whereas 'no serum' and 'serum 1/100' had initial high viability which decreased by 48 hours. There was no significant difference between serum diluted 1/100 and no serum ($p=0.97$).

**Table 7-2. Averaged percentages of cell viability from triplicates of cell lines.**

| | | Viability (%) | |
|---|---|---|---|
| **Time point** | **Serum dilution** | **MO3.13** | **SY5Y** |
| ***6 hours*** | **No serum** | 95.7 | 97.6 |
| | **serum 1/100** | 95.2 | 97.8 |
| | **serum 1/1000** | 84.4 | 83.9 |
| ***24 hours*** | **No serum** | 96.0 | 96.7 |
| | **serum 1/100** | 93.3 | 97.2 |
| | **serum 1/1000** | 92.3 | 82.5 |
| ***48 hours*** | **No serum** | 93.7 | 77.2 |
| | **serum 1/100** | 90.3 | 77.0 |
| | **serum 1/1000** | 89.3 | 87.3 |

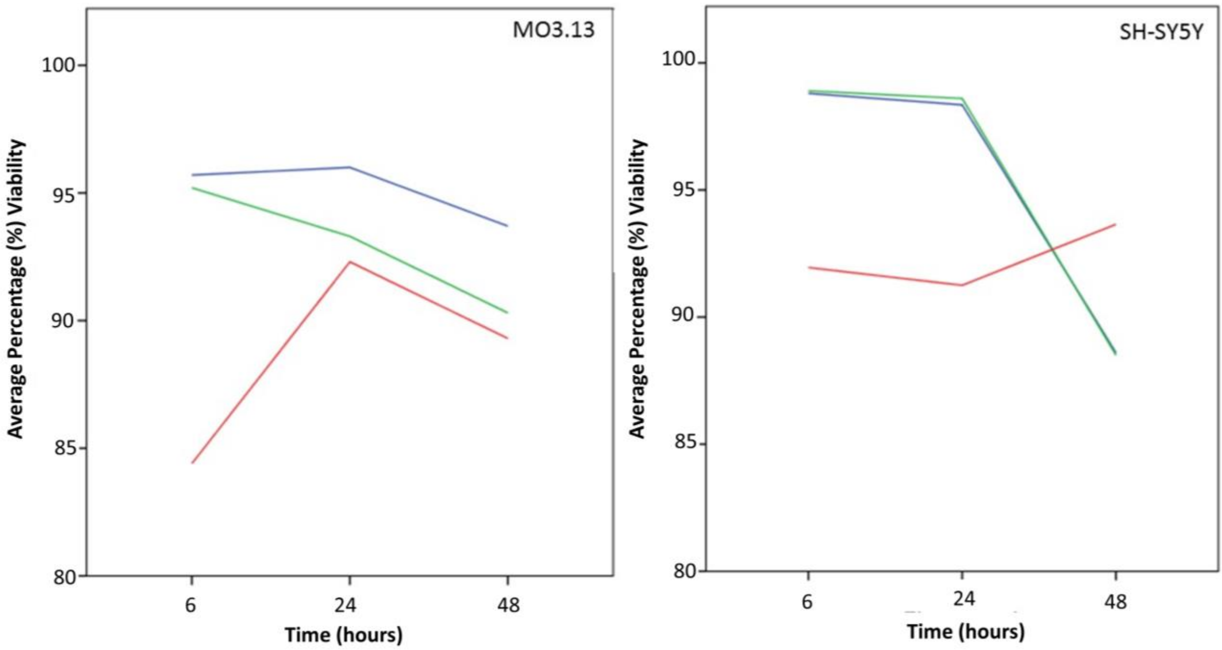


**Figure 7-4. Average viability of MO3.13 and SH-SY5Y cell lines with and without MS serum over 48 hours.** Overall, there are no significant changes in viability between no serum (blue), serum diluted 1/100 (green) and 1/1000 (red) for either cell type.

## 7.3. Discussion

### 7.3.1. Functions of proteins identified by hexSelect macroarray

From our previous studies presented in past chapters, we identified that MS patients have more antibodies (IgG) against the EBNA-1$_{(398\text{-}413)}$ peptide of EBV. Here we aimed to improve our knowledge of antibody-mediated MS pathogenesis based upon the possible cross-reactive and functional capabilities of those antibodies. Furthermore, we wanted to identify whether the isolated antibodies that independently contribute to a risk model can target any human brain proteins identified by macroarray. Both MS patient sera and control plasma reacted against multiple proteins of the array, confirmed in duplicate reactivity against the same protein. A summary of the known functions of the identified reactive proteins is given in Table 7-3. The fact that reactive proteins were identified with control plasma is not surprising, as it is well established that a healthy immune system contains antibodies against MBP and αßC, and possibly other brain proteins. Pooled serum from acute and progressive MS individuals did bind in part to the same proteins as seen for healthy controls, but additionally showed binding to different proteins. Progressive MS cases only showed reactivity to two targets, whereas acute cases showed reactivity to seven targets (with STMN4 having four separate duplicates). However, it is not known whether these are exactly the same target or different sections of the whole protein. It was unexpected that there were not more shared targets between the different MS clinical subgroups, but whether this is related to the disease progression remains unclear.

**Table 7-3. Function of identified proteins confirmed by macroarray.**

| Role | Protein Name | Function in humans | A-MS | P-MS | HC |
|---|---|---|---|---|---|
| *DNA/ transcription* | CENPB | Interacts with heterochromatin, believe to assist organization of DNA structure and centromere formation. | | | ✓ |
| | MBD3 | Transcriptional repressor, role in gene silencing, although doesn't bind to DNA by itself. | | | |
| | JMJD8 | Hydroxylating/demethylates proteins▲ | ✓ | | ✓ |
| | SHB2 | Regulates signal transduction cascades by linking activated receptors to downstream signalling components. May play a role in angiogenesis, regulating insulin-producing cells, TCR & IL-2 signalling, apoptosis and neuronal cell differentiation. | ✓ | | |
| | EEF1A2 | Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis | ✓ | | |
| | HDAC5 | Deacetylates lysine residues of core histones, which relates to transcriptional regulation. | ✓ | | ✓ |
| | TROVE2 | Binds to misfolded non-coding RNA, stabilizing and protecting them from degradation. | | ✓ | |
| | NFKBIL2 | Also known as TONSL, "tonsoku like protein", part of complex which maintains genome integrity during DNA replication. | | ✓ | |
| | KAT2A | "General control of amino acid synthesis protein-5 like 2." Promotes transcriptional activation. | | | ✓ |
| *Growth control* | PIM3 | Prevents apoptosis, promote cell survival and protein translation. Involved in control of energy metabolism and regulation of cell growth. | ✓ | | |
| *Metabolism/ Energy* | AMPD2 | Critical role in energy metabolism, part of purine nucleotide cycle. | | | ✓ |
| | AZGP1 | Stimulates lipid degradation in adipocytes, may bind polyunsaturated fatty acids. | | | ✓ |
| *Blood* | HBA2 | Role/importance not known, but haemoglobin carries oxygen for red blood cells. | ✓ | | |
| *Autophagy* | MAP1LC3A | Involved in formation of autophagosomal vacuoles (autophagosomes). | | | ✓ |
| *Endocytosis* | EPN1 | Regulates receptor-mediated endocytosis. | ✓ | | |
| *Microtubule* | STMN4 | Microtubule destabilizing activity. | ✓ | | ✓ |
| *Unknown* | UFC1 | Forms intermediate with UFM1, which role is not yet known. | | | ✓ |

Functions from as summarized in (747). ▲Role as reported by (748). A-MS: acute MS. P-MS: progressive MS. HC: healthy controls.

The most surprising result from this experiment was that targets for isolated anti-EBNA-1$_{(398-413)}$ antibodies were not also seen for pooled MS serum from the same patients, and that repeating the array with the same pooled plasmas of healthy controls showed additional results with only partial confirmation of targets identified when used the first time. Possibilities for these results could be attributed to either (i) specificity, in that non-specific binding is occurring between antibodies and proteins and so the duplicate results are "false positive", or (ii) sensitivity of the array is not allowing detection of "true" binding of antibodies to the proteins, which would explain the lack of confirmation in replication (Healthy plasma [2]). Either rationale could be confirmed by western blot. In western blots either isolated antibodies or serum are tested for reactivity against a single candidate protein. Interestingly, no antibodies were identified as cross-reactive to myelin or αßC, as expected for anti-EBNA-1$_{(398-413)}$ antibodies due to sequence homology (as reviewed in Chapter 5). The protein array did contain several similar proteins (beta-crystallin B2, sphingomyelin phosphodiesterase, myelin transcription factor 1, myelin expression factor 2, myelin protein zero-like protein 1 & 3 precursor and myelin transcription factor 1-like protein), but reactivity was not identified using our assay. However, the exact protein sequences of proteins used for the array are not known, and so homology with anti-EBNA-1$_{(398-413)}$ is speculative and cannot be more closely evaluated. Some proteins were identified to show reactivity within healthy controls and MS patients (STMN4 and HDAC), which have transcriptional and structural roles.

Interestingly, only acute MS samples reacted to HBA2 and EPN1, related to blood regulation and endocytosis respectively, and PIM3, related to cell growth control. Only healthy control samples reacted with targets associated with metabolism (AMPD2 and AZGP1). The healthy controls did show more reactivity, but also had more individuals included in the pooled samples than MS (ten compared to three). The protein macroarray did not result in the anticipated large

number of reactivities. Therefore, the reported reactive proteins should be individually confirmed by western blot, separating the samples per patient or single control. This is also important as the proteins expressed on the macroarray cannot be confirmed by the company to have human post-translational modification, and so may miss some cross reactive targets due to conformational differences to the *in vivo* situation. Western blot confirmation is of particular importance for proteins not included on the macroarray such as αßC, which is known to have sequence homology with the EBNA-$1_{(398\text{-}413)}$ epitope. The differences in reactivity between progressive and acute MS could represent the deterioration of the immune system with disease progression, although cellular experiments on these patients would additionally shed light on the involvement of immune dysregulation.

### 7.3.2. Effect of patient sera on cell culture viability

The key limitation on *in vitro* models for MS has been the heterogeneity and complexity of the adult human CNS, which has multiple interacting cell types. Elliott *et al* (728) overcame this limitation by using a rat brain model, which although impressive through its combination of homologous growth factors and modelling structure, still leaves a gap of translation between the animal model and humans *in vivo*. The cell lines we obtained are both human derived, and the use of the SH-SY5Y cell line in itself acts as a good control as it is sourced from neurons of the peripheral nervous system, compared to MO3.13 which is derived from oligodendrocytes from the CNS, which are more readily targeted in disease pathogenesis. Ideally, a cell culture model using oligodendrocytes, astrocytes, neurons and glial cells of mature human brain would be used. As cells are dependent on neighbouring cells and their produced molecules, a 3D model would be highly advantageous. Some groups are working towards this, however complications arise from the competitive growth rates of some cells impeding expansion of others, and so accomplishing the physiological ratio similar to *in vivo* is difficult.

In this study, we were able to create differentiated oligodendrocyte cell lines, although defining them as terminated would be premature, as more than a single chemical agent is needed for differentiation *in vivo*. Both cell lines were confirmed as Mycoplasma negative as infection can alter the cell's metabolism and could lead to misleading results. Preliminary evaluation of the cell lines showed that no effect of serum was seen in the MO3.13 cell line over time. The decreased viability at 48 hours may have been due to the cells' immature state and rapid growth, limiting nutrients and causing a higher death rate. As for SH-SY5Y, there is no clear reasoning for why serum 1/1000 did not follow the same decreased viability at 48 hours, although it could be possible that there were beneficial components in diluted patient serum, in opposition to the postulated hypothesis. For both cell lines, it is plausible that the serum was too dilute to achieve any antibody-dependent cytotoxicity without cells or additional complement added to the cells. Pooling serum could address this issue, as well as investigating mature cell forms where the growth rate isn't as rapid. These cell cultures will be useful for future studies which could test patient derived antibodies in combination with patient derived immune cells, even isolated EBV-infected B cells, to review their *in vitro* capabilities as representative of the *in vivo* microenvironment. With antibodies contributing to ADCC, opsonisation, complement activation and activation of effector cells (749), the true benefit of the antibodies may need to be seen in context of patient immune cells, and their combined effect.

## 7.4. Summary

Immune cross-reactivity has long been proposed as an important component in MS pathogenesis, whether as a trigger or contributing factor. With a large amount of evidence supporting IgG antibodies against EBV as a necessary prerequisite to developing MS, in particularly EBNA-1, finding cross-reactive targets of these elevated antibodies is of great interest. Results from this study showed that healthy control and MS serum contained IgG antibodies which bound to a range of different brain proteins involved in several signalling pathways with a range of functional roles. Some proteins were common between healthy controls and MS samples, but most were unique to either group. A higher background was seen when testing serum rather than isolated antibodies on the hexSelect macroarray, however isolated antibodies were cross reactive to a short epitope within EBNA-1 and human brain proteins. Healthy control samples contained more reactive proteins compared to MS samples and protein reactivity was different within different clinical patterns of MS. Cell cultures were successfully grown in the laboratory and proven negative for *Mycoplasma*. Both cell lines showed an overall non-significant influence of serum on viability, however this could be due to their immature state or the level of serum. Further experiments could include binding potential of anti-EBNA-1$_{(398-413)}$ antibodies to these cell cultures which could be checked with confocal microscopy, as well as the influence of patient-derived EBV-specific or EBV infected immune cells on these cells lines at different differentiation stages *in vitro*.

# 8. General Discussion

MS is a multifaceted disease, with several risk factors. Therefore treatment of the cause for inflammation and demyelination is challenging. The role of EBV infection in MS as a contributing risk factor is not yet fully understood. The focus of this project was to identify how adaptive immune responses against EBV differ between MS patients and healthy controls, with particular focus on a known B cell epitope within EBNA-1. To the author's knowledge, this is the first study of EBV serology using these two well-established Western Australian cohorts. The MS cohort in our study reflects the global gender bias, with a 3:1 prevalence of females, while the ratio of gender in the healthy cohort was approximately 1:1. Additionally, over half of the individuals in the MS cohort carried "high-risk" HLA-DR alleles, according to grouping by Nolan *et al* (184), while just over half of participants in the control group carried "low-risk" HLAs. This is in line with the concept that there is a genetic predisposition for development of MS, upon which environmental factors further increase the risk, and perhaps 'trigger' MS.

Associations between EBV-specific immunity and MS pathogenesis can be grouped into two schools of thought; (i) naturally occurring autoreactive B cells are latently infected with EBV, evade elimination due to the viral immune evasion and/or inadequate immunological control, and consequently expand, or (ii) autoreactive T (and maybe B) cells develop through persistent targeting of EBV-specific proteins, potentially through HLA-restricted antigen presentation and antibody-dependent mechanisms, leading to viral-host cross-reactivity. Both theories have supporting evidence as discussed by Pender *et al.* (377) and outlined in Chapter 1.5.6, but one should consider that these theories might not be mutually exclusive. They have evolved with new knowledge of MS pathogenesis, and should continue to do so. Figure 8-1, created by the author of this thesis, depicts how MS pathogenesis can be caused by multi-faceted immune responses against EBV and particularly EBNA-1, with primary focus on the virus' role in causing inflammation in the CNS through cross-reactivity. This model integrates novel aspects

into past theories of MS mechanisms such as T cell only cross-reactivity as the model also includes the important role of B cells in the pathogenesis, and incorporates the ideas of bystander-damage. Additionally, it supports the hypothesis that EBV-specific cross-reactivity with αβC results in inflammation within the CNS (steps 18 and 19 in Figure 8-1). The model also includes considerations of the role of autoreactive T cells in MS pathogenesis as outlined in steps 1, 5 and 15b and that EBV and EBV-infected B cells have been identified in post-mortem CNS tissue (324, 500, 513, 531-533) as shown in step 2. The data presented in this thesis includes a targeted investigation of EBV-specific antibodies, produced by specialized plasma cells in response to EBV infection. In particular, experimental results of this thesis add to the current understanding of MS pathogenesis in steps 8, 11, 12 and 17. Primarily, EBNA-1 specific plasma cells produce anti-EBNA-1 IgG antibodies with the potential to cross-react with brain proteins when present in the CNS.

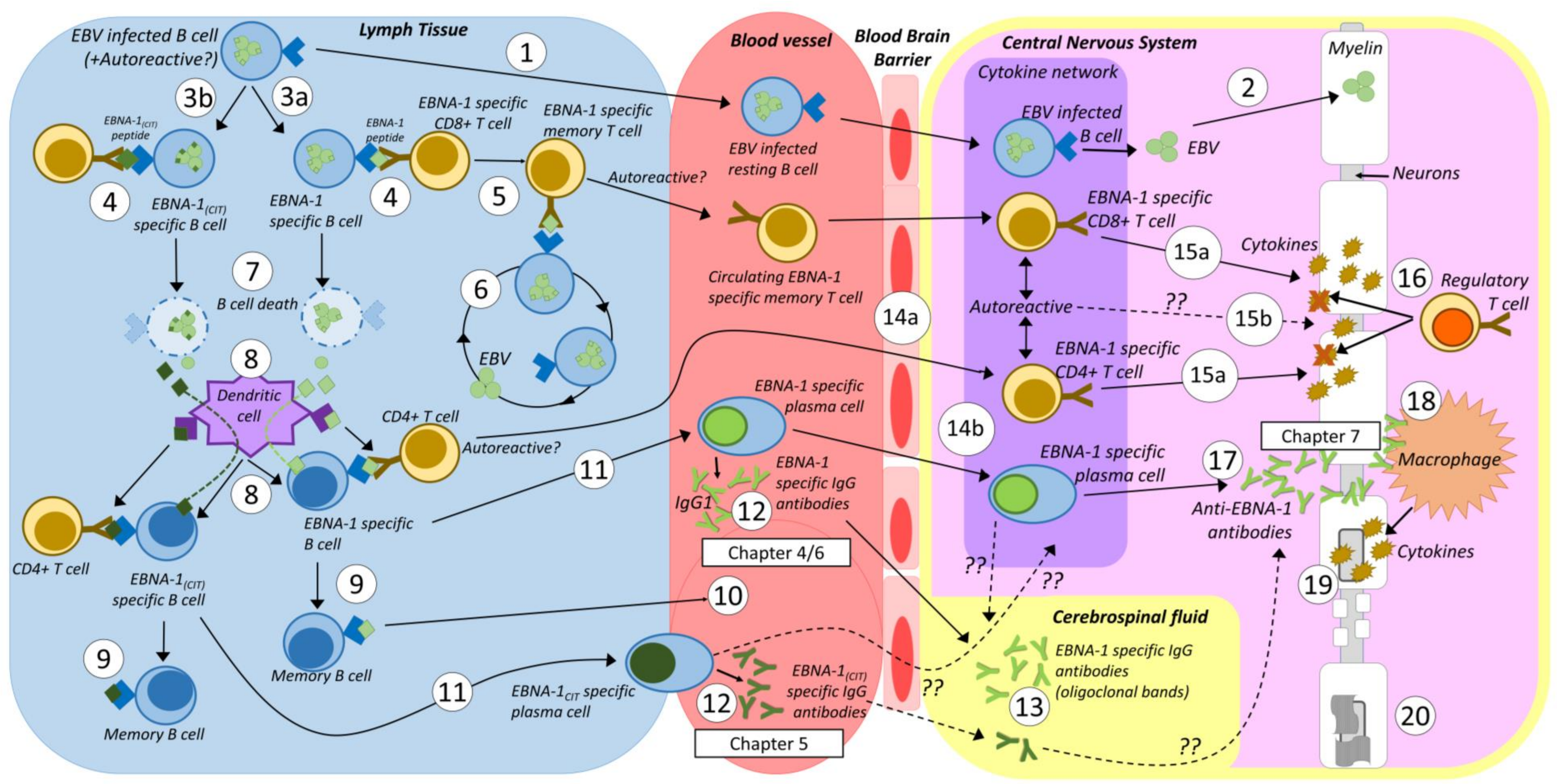


**Figure 8-1. Model of MS pathogenesis involving EBV infection**. [EBV: Epstein-Barr Virus. EBNA-1: Epstein-Barr Virus Nuclear Antigen. IgG: Immunoglobulin G. TCR: T cell receptor. HLA: Human Leukocyte Antigen. NK: Natural killer cell. aa: amino acids.] (1) EBV infected B cells (autoreactive or EBV specific) evade immune recognition and enter the CNS. (2) EBV infected B cells can reactivate within ectopic follicles in the CNS and produce EBV, which can infect other B cells and possibly other cell types (e.g. epithelial, NK and T cells). (3a) EBV infected B cell presents EBNA-1 peptide to immune system. (3b) Citrullinated EBNA-1 (dark green) peptide is presented to the immune system. (4) EBNA-1 presenting B cells are recognized by CD8+ T cell specific for EBV through TCR; recognizing EBV peptide (8-10aa) presented through class I

HLA. (5) These CD8+ T cells become memory T cells. (6) Memory T cells can be re-stimulated by new EBV presenting B cells, as EBV goes through stages of replication to infect more cells. (7) B cells are eliminated by CD8+ T cell recognition, cells break apart and release EBV peptides. (8) Peptides are taken up by dendritic cells which independently present or assist presentation of EBNA-1 peptide (approx. 15aa) through B cells to CD4+ T cells, through class II HLA. (9) Following B cell activation by CD4+ T cells, B cells can mature into memory B cells. (10) These memory B cells can enter circulation. (11) B cells can also mature to become plasma cells, which enter the peripheral system. (12) Plasma cells release antibodies specific for the antigen presented by the B cell, which with environmental triggers, class switch into IgG. (13) These antibodies can cross the disrupted blood brain barrier (BBB) and enter the cerebrospinal fluid, as identified in oligoclonal bands. (14a) CD8+ and CD4+ T cells as well as plasma cells can cross the disrupted BBB and enter the CNS. (14b) Within the CNS, cells release cytokines and chemokines, affecting surrounding cells, promoting more inflammation and recruitment of more immune cells. (15a) EBNA-1 specific T cells attack myelin and/or oligodendrocytes through cross-reactivity. (15b) These T cells could also be autoreactive, primed by EBV-infected autoreactive B cells, and thus attack myelin and oligodendrocytes. (16) Regulatory T cells release anti-inflammatory cytokines to limit demyelination. (17) EBNA-1-specific plasma cells produce EBNA-1-specific antibodies, which cross react with brain proteins. (18) Fc of cross-reactive anti-EBV IgG antibodies are recognized by Fc receptor of macrophages and initiate damage of self (e.g. myelin and oligodendrocytes). (19) Macrophages demyelinate neurons, releasing potentially more cross-reactive antigens and perpetuate inflammation through cytokine release. (20) Partially effective remyelination occurs.

This thesis used optimized automation for all immunoassays to ensure accurate quantitation of anti-EBV antibodies for subsequent analysis. Using ELISA techniques, is was shown that the MS cohort was universally positive for EBV infection, whereas only 90% of the controls were EBV seropositive. This is in line with the literature (341, 449, 563), and EBV infection is a prerequisite for our model in Figure 8-1, as EBV infection is necessary for autoreactive B cells to evade immune elimination, and for EBV-specific B cells to continually stimulate the EBV-specific T cell population (step 5 of Figure 8-1), with both cell populations being able to trigger cross-reactivity in the CNS (steps 15 and 17). Our MS cohort had significantly higher IgG antibody titres against VCA and EBNA-1 in concordance with the vast majority of studies (545, 573). These elevated antibody levels could indicate a higher risk of cross-reactivity with self peptides by sheer titre of antibodies, as well as indicating that T cells might not be providing adequate control of EBV reactivation. Gender and age were seen to influence immune response to EBNA-1 and VCA, however the association of HLA and antibody titres is most intriguing. In logistic regression analysis, high-risk HLA significance was abrogated by inclusion of EBNA-1 and VCA ELISA data, suggesting a common pathway. This genetic association with EBV infection warrants further research, potentially through the modelling of EBNA-1 and VCA peptides and presentation by “high-risk” HLA compared to “low-risk” HLA alleles.

Extrapolating from the publication by Mechelli *et al.* (582) which studied MS-discordant identical twins, a novel in-house ELISA was developed and optimized to investigate IgG antibodies against a short B cell epitope within the EBNA-1 protein: EBNA-$1_{(398\text{-}413)}$. Significantly more samples were seropositive in the MS cohort and had higher antibody titres against EBNA-$1_{(398\text{-}413)}$ than healthy controls. This reflects that EBV is a requirement for developing MS as well as the possibility that EBV-specific plasma cells are higher in number and/or reactivity in MS patients. It is notable that another study targeting an epitope within

EBNA-$1_{(398-404)}$ (581) did not find significant differences between MS and healthy controls (Figure 4-7). Their epitope was only six amino acids long, thus highlighting the importance of optimal peptide length to present a real antibody target as well as emphasizing the immunogenicity of the EBNA-$1_{(398-413)}$ peptide used in this thesis, and its significance in immune responses in relation to MS risk. Although not associated with HLA-DR, gender or age, this novel experiment adds to existing understanding of EBNA-1 peptide immunogenicity with antibodies against this peptide significantly improving the logistic regression model (Figure 4-6), increasing the discrimination between MS cases and controls, in addition to the commercial anti-EBNA-1 ELISA results. As mentioned in Chapter 4.3, validation of this model with another cohort would be most beneficial, either with another Australian cohort, a cohort from the Northern Hemisphere, or from a population with different HLA MS risk alleles (162, 163). Our antibody findings indicate presence of EBNA-$1_{(398-413)}$ reactive B cells in patients that mature into plasma cells as shown in steps 8 and 11 in Figure 8-1, which could reflect poor control of EBV infection and the ability to restimulate T cells responses. This should be examined further through characterizing EBV-specific T cell populations, which our group is currently investigating. Additionally, the EBNA-$1_{(398-413)}$ peptide shares homology with αßC, which is present in the CNS and could be evidence of molecular mimicry as a cause of cross-reactivity, similar to Lünemann's finding of EBNA-1 specific T cells cross-reacting with myelin (480), as described in step 15a of Figure 8-1.

Data on the anti-EBNA-$1_{(398-413)}$ IgG antibody subclasses was the first of its kind, following from observations of enriched EBV-specific IgG1 antibodies in MS cases previously published by Lünemann *et al.* (480) from a small set of patients and controls. Surprisingly, anti-EBNA-$1_{(398-413)}$ IgG1 antibodies were detected in less than half of each cohort, and anti-EBNA-$1_{(398-413)}$ IgG2 and IgG3 in less than 10%. This path of research may therefore offer

value in terms of revealing the functional characteristics of EBNA-1-specific antibodies in the context of MS pathogenesis as part of our MS model, such as opsonisation or recruitment of other cells. Importantly, we were able to detect anti-EBNA-$1_{(398-413)}$ IgG3 antibodies using our in-house ELISA, which had never been published before. These antibodies contributed to a reduced MS risk in the logistic regression model and are of high importance. Indeed, the anti-EBNA-$1_{(398-413)}$ IgG1 and IgG2 elevation in MS cases did not remain significant in the logistic regression while IgG3 did. This could reflect that in response to a sustained pro-inflammatory environment, more IgG antibody subclass switching from IgG3 to IgG1 occurs in MS patients.

EBV specific antibodies within oligoclonal bands in the CSF have been identified previously (476, 478, 572, 583, 584, 586, 587). Following the significance of anti-EBV antibody quantitation, this thesis showed that anti-EBNA-$1_{(398-413)}$ antibodies which are present in MS and healthy controls have the ability to cross-react with brain proteins involved in a range of functions. This experiment was the first of its kind. The potential pathogenic role of these antibodies is shown in step 17 of Figure 8-1: presence of anti-EBNA-1 antibodies in blood and CSF as well the ability of some antibodies to cross-react with proteins within the CNS, causing antibody-dependent cytotoxicity, in addition to damage from T cells. Autoreactive antibodies were identified in both MS patients and healthy controls for a range of proteins. There was little overlap of common targets (Table 7-3), with only MS serum showing reactivity for proteins involved in endocytosis (EPN1) and growth control (PIM3) while only healthy controls showed reactivity for proteins associated with metabolism (AMPD2 and AZGP1). This shows that autoreactive antibodies are common in healthy controls, potentially from EBV-infected autoreactive B cells but quantity of antibodies may differ between cohorts. There were shared targets for different proteins involved in the DNA/transcription pathway. The crucial difference is that in MS patients, these autoreactive antibodies have the ability to cross the disrupted blood-

brain barrier into the CSF and CNS, identify targets not presented in the periphery and contribute to inflammation. This is supported by the presence of anti-EBNA-1 reactive IgG in MS CSF (572). When testing isolated anti-EBNA-$1_{(398\text{-}413)}$ antibodies, we detected two novel cross-reactive targets: haemoglobin subunit alpha (HBA2) and elongation factor 1-alpha2 (EEF1A2) which have roles in blood and DNA/transcription pathways, respectively. These might be additionally involved in MS pathogenesis through their pathways to inhibit new myelin production (steps 19 and 20 of Figure 8-1) and affect oxygen delivery to the microenvironment. Surprisingly, isolated antibodies and matched serum did not detect the same targets on the macroarray. This could be due to a range of factors including (i) different binding affinity of antibodies, (ii) the presence of inhibiting co-factors within the serum or (iii) relatively lower levels of epitope specific antibodies to other autoreactive antibodies in serum. By utilizing anti-EBNA-$1_{(398\text{-}413)}$ antibody isolation, autoreactive antibodies were further enriched and so protein targets could be identified that were masked by using whole serum. This should be considered in future serum and/or CSF testing of MS samples for cross-reactivity. The benefit of the protein macroarray is that it allows a multitude of potential targets to be screened, and we next plan to confirm the cross-reactivity of identified targets by Western blot. For future cross-reactivity testing of anti-EBNA-$1_{(398\text{-}413)}$ antibodies, inclusion of MBP, GFAP and αßC proteins which were not present on the commercial macroarray (listed in Chapter 7.4) should be included due to their advocated immunogenicity in MS. In particular, cross-reactivity with αßC should be further investigated as it shares sequence homology with our EBNA-1 peptide. This cross-reactivity of isolated anti-EBNA-$1_{(398\text{-}413)}$ IgG support our model of EBV-specific immune cross-reactivity contributing to inflammation and neuronal degradation as shown in step 17 of Figure 8-1.

Citrullinated autoantibodies have a prominent role in RA diagnostics, therefore we also examined the role of citrullination of the EBNA-1$_{(398\text{-}413)}$ peptide. No literature to date has investigated whether anti-EBV responses are altered by this pro-inflammatory modification in MS, or in relation to different HLA alleles. The model in Figure 8-1 incorporates this, showing the processing of citrullinated epitopes through B and T cell interactions, resulting in anti-citrullinated EBNA-1 antibodies. In this study, MS cases had significantly higher EBNA-1$_{(398\text{-}413\ CIT)}$ seropositivity and elevated antibody titres compared to controls, although no influence by HLA-DR, gender or age was found. Although anti-EBNA-1$_{(398\text{-}413\ CIT)}$ IgG levels in MS were not as discriminatory as citrullinated autoantibodies in RA, they did independently contribute to the risk model in Chapter 5, and likely reflect a larger sustained pro-inflammatory environment in MS patients. It would be intriguing to investigate whether MS CSF oligoclonal bands are reactive to citrullinated EBNA-1 peptide as has also been shown for non-citrullinated forms (584). Immunofluorescence experiments on post-mortem brain samples could further establish if these antibodies are present in the CNS. For future study, cross-reactivity of EBNA-1$_{(398\text{-}413\ CIT)}$ IgG and brain proteins would be of high interest as MBP and homologous αßC are both known to become citrullinated. A study by Svendsen *et al* suggested a genetic component to autoantibodies in RA-discordant twins (750), which could be investigated in our MS cohort to identify whether MS citrullinated antibodies reflect an autoreactive or a modified anti-EBV immune response through cross-reactivity (steps 12 and 13 of Figure 8-1). Additionally, the effect of citrullinated EBV antibodies could be explored using a mouse model such as EAE (described in Chapter 7.1.3). In EAE, immunization with myelin antigen induces neurological damage in a short time frame, and allows for identifying T cell-mediated mechanisms following immunization. However, the role of herpesviruses in mouse EAE pathogenesis has been only recently investigated (751). Primate studies would be even more informative as recent studies have highlighted the role of EBV-infected B cells in

disease progression, and myelin immunization to cause MS-like symptoms might be triggered through different pathways (752). Despite not having mouse or primate models, this thesis did functionally investigate the potential effect of these antibodies on oligodendrocytic and neuronal cell lines *in vitro.* Whole serum of MS patients did not show a pathogenic effect on viability for either cell line in preliminary experiments, but this does not mean the antibodies are ineffective. These results could be explained by high serum dilution levels, the replicative ability of cells in undifferentiated forms. Furthermore, these high antibodies could have a long-term cytotoxic effect, or inclusion of other cellular components such as T cells, B cells and/or macrophages are needed to reproduce MS pathogenesis *in vitro* as shown in step 18 and 19 in Figure 8-1. Additionally, other than causing direct apoptosis, EBV specific antibodies could lead to damage of brain proteins by recruiting other cells, changing the chemokine environment and up/down regulating pathways involved in cell signalling.

Future aims of MS research should focus on the importance of EBV, and include investigations of the cellular response and control of the virus, to contribute a better understanding to the model shown in Figure 8-1, and extend from epidemiological studies to functional aspects of the pathology. This important concept could recently be shown in a proof of principle study by Pender *et al.*(624). The group isolated patient-derived EBV-specific T cells and saw a beneficial clinical outcome when reintroducing these cells into a SPMS patient after enrichment. To further investigate this, our group plans to immunophentoype EBNA-1 specific CD4+ and CD8+ T cell subpopulations and to investigate their cytokine profiles in MS patients. Additionally our group is planning to study the role of regulatory T cells, as depicted in step 16 of Figure 8-1, as they represent the ability to suppress inflammation and low regulatory T cells could be an additional mechanism by which MS pathogenesis occurs. It would also be very interesting to further investigate this MS study population, with regard to when EBV infection

occurred or if they had IM, as IM has been associated with MS risk (384, 452-456) in addition to HLA-DRB1*15 (458) and latitudinal distribution (457). How IM is associated with MS is not yet fully understood, but it is possible that primary infection with EBV later in life results in a more mature immune response, and this increases MS risk by how well the virus is controlled. Lastly, the influence of UVB and vitamin D should not be underestimated in their roles in the promotion of autoimmune diseases (outlined in chapter 1.4.3), and of MS more specifically. In this regard, it would be interesting to investigate whether individuals within the MS cohort migrated to Australia, and if it was early or later in life. This could benefit through identifying additional factors such as early-life or cumulative sunlight exposure as risk factors for MS, and to assess how these factors relate to our existing statistical model.

The epidemiological study presented in this thesis offers new insights into the contribution of EBV to MS, showing anti-VCA and particularly anti-EBNA-1 IgG as well as combined HLA-DR alleles and gender can be integrated into a significant risk model. This study is the first of its kind to identify significantly elevated antibodies in MS specific for EBNA-$1_{(398-413)}$ IgG, and identified a subclass bias towards IgG1 in MS using this target. Antibodies specific for citrullinated EBNA-$1_{(398-413)}$ were not as common as uncitrullinated targets, but significantly contributed to MS risk, suggesting this modification may be involved in separate mechanisms for causing damage. Lastly, this study identified novel cross-reactive brain protein targets for EBNA-$1_{(398-413)}$ specific antibodies. This provides the conceptual framework for functionality of these antibodies, from which we can further investigate cell populations and signalling pathways to elucidate other mechanisms behind triggering and progression of MS. These findings support the pathogenic importance of EBV in MS development, and its potential for diagnostic and therapeutic targeting that can be applied in clinical practice in order to improve

patient outcome and in the future aid in identifying at-risk individuals to implement preventative measures.

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# 10. Appendix I: Publications and Presentations

## 10.1. Publications

Strautins, K., M. Tschochner, et al. (2014). "Combining HLA-DR risk alleles and anti-Epstein-Barr virus antibody profiles to stratify multiple sclerosis risk." Mult Scler **20**(3): 286-94.

## 10.2. Presentations

K. Strautins, M. Tschochner, C. Berry, I. James & D. Nolan. Investigating Epstein-Barr virus (EBV) antibodies in Multiple Sclerosis patients, and development of an anti-EBV nuclear antigen-1 B cell epitope ELISA. Australian Society for Medical Research (ASMR) Scientific Symposium. **7 June 2012.** Perth, Western Australia. [Presentation Prize]

K. Strautins, M. Tschochner, C. Berry & D. Nolan. Development of an anti-Epstein-Barr Virus nuclear antigen-1 ELISA and investigation of B-cell-epitope specific antibodies in Multiple Sclerosis patients. Murdoch University Postgraduate Student Association (MUPSA) Multidisciplinary Conference. **27 September 2012.** Perth, Western Australia.

K. Strautins, M. Tschochner, C. Berry, D. Nolan. Epstein-Barr virus (EBV)-specific antibodies in multiple sclerosis patients, including targeted investigation of a candidate EBV nuclear antigen-1 (EBNA-1) B cell epitope. 28th Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS). **10-13 October 2012**. Lyon, France.

K. Strautins, M. Tschochner, C. Berry, I. James & D. Nolan. Stratifying Multiple Sclerosis risk utilizing Epstein-Barr virus antibody profiles, genetic risk factors, gender and a novel anti-EBV

nuclear antigen-1 B cell epitope ELISA. Australian Society for Medical Research (ASMR) Scientific Symposium. **5 June 2013.** Perth, Western Australia.

K. Strautins, M. Tschochner, I. James, L. Choo, DS. Dunn, M. Pedrini, A. Kermode MD, W. Carroll & D. Nolan. *Combining HLA-DR risk alleles and anti-Epstein-Barr Virus antibody profiles to stratify Multiple Sclerosis risk.* Invited Seminar at Institute for Immunology and Infectious Diseases (IIID). **23 April 2013.** Perth, Western Australia.

K. Strautins, M. Tschochner, C. Berry, I. James & D. Nolan. *Multiple Sclerosis risk assessment: combining Epstein-Barr Virus antibody reactivity with genetic risk factors.* Murdoch University Postgraduate Student Association (MUPSA) Multidisciplinary Conference. **3 October 2013.** Perth, Western Australia.

K. Strautins, M. Tschochner, C. Berry, I. James & D. Nolan. *Influence of gender, genetic factors and Epstein-Barr Virus antibody profiles, on Multiple Sclerosis risk.* 23rd Annual Combined Biological Sciences Meeting (CBSM). **30 August 2013**. Perth, Western Australia.

K. Strautins, M. Tschochner, I. James, L. Choo, DS. Dunn, M. Pedrini, A. Kermode MD, W. Carroll & D. Nolan. Combining Genetic Risk Factors, Gender And Anti-Epstein Barr Virus (EBV) Antibody Profiles, Using A Novel Anti-EBV Nuclear Antigen-1 (EBNA-1) B-Cell-Epitope ELISA, To Stratify Multiple Sclerosis Risk. 6th Congress of the Pan-Asian Committee for the Treatment and Research in Multiple Sclerosis (PACTRIMS). **6-8 November 2013**. Kyoto, Japan.

K. Strautins, M. Tschochner, C. Berry, I. James & D. Nolan. *Adaptive immune responses against Epstein-Barr Virus influencing Multiple Sclerosis risk.* 24th Annual Combined Biological Sciences Meeting (CBSM). **29 August 2014**. Perth, Western Australia.

K. Strautins, M. Tschochner, C. Berry, I. James, S. Leary, L. Choo, H. Clark, A. Chopra, D. Cooper, A. Kermode, W. Carroll & D. Nolan. *Investigating the role of EBV infection in Multiple Sclerosis pathogenesis.* 1st Science on the Swan conference. **21-23 April 2015.** Perth, Western Australia. [Poster prize]

K. Strautins, M. Tschochner, I. James, L. Choo, DS. Dunn, M. Pedrini, A. Kermode MD, W. Carroll & D. Nolan. *Investigating the role of Epstein-Barr Virus in the pathogenesis of Multiple Sclerosis.* Invited Seminar at Institute for Immunology and Infectious Diseases (IIID). **10 June 2015.** Perth, Western Australia.

# 11. Appendix II: Supplementary Figures and Tables

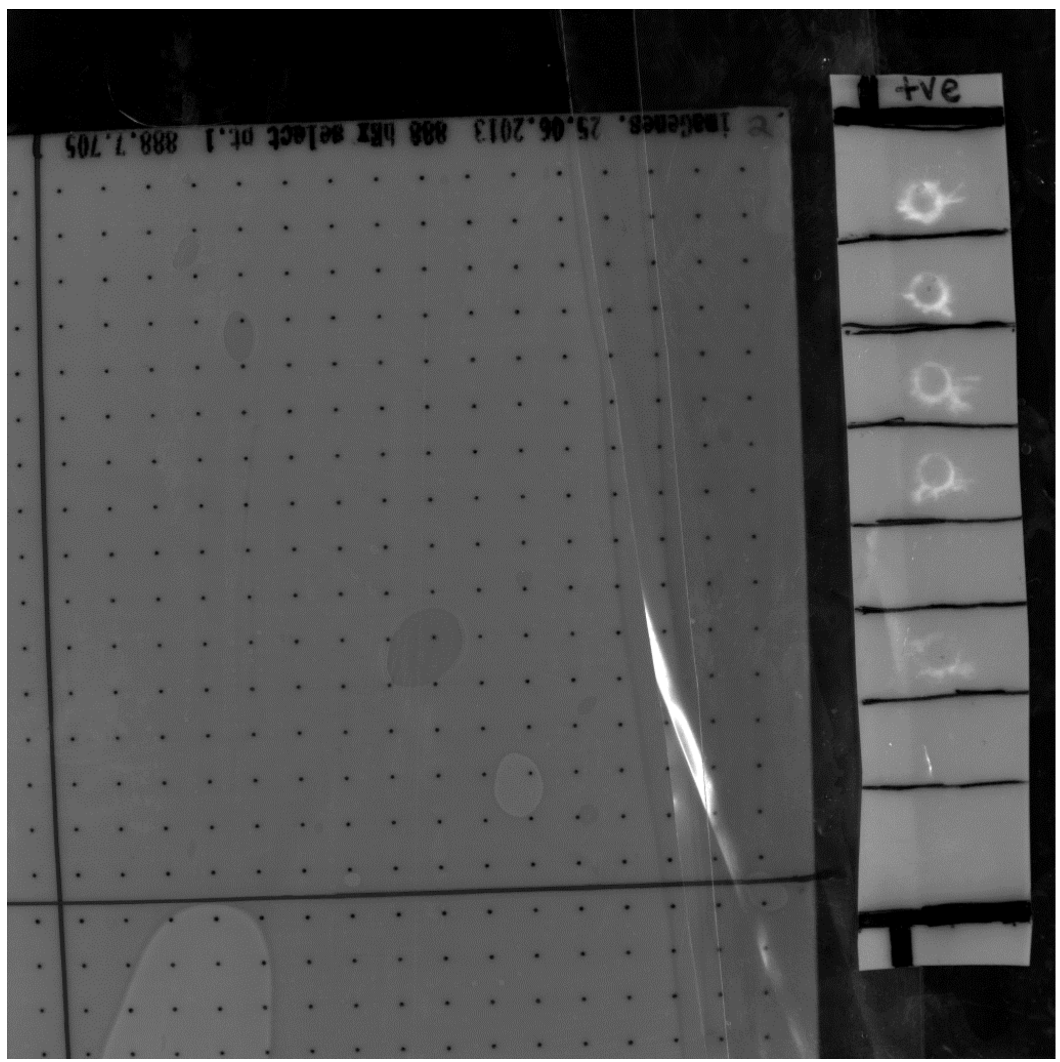
**Supplementary Table S-1. All Reagents and Kits Catalogue Reference Numbers.**

| Reagent | Company | Catalogue Number |
|---|---|---|
| **10X PCR buffer** | Invitrogen | Y02028 |
| **1Kb Plus DNA Ladder** | Invitrogen | 10787-018 |
| **24 well Tissue Culture Plate with lid** | Becton Dickinson | 353047 |
| **3, 3', 5, 5' Tetramethylbenzidine (TMB)** | Sigma Aldrich | T4444 |
| **Ampure Purification Kit** | Beckman Coulter | A63882 |
| **Antibiotic-Antimycotic (100X)** | Invitrogen | 15240-096 |
| **beta-mercaptoethanol** | Sigma Aldrich | M7522 |
| **Bovine Serum Albumin (BSA)** | Sigma Aldrich | A7906 |
| **CleanSeq Purification Kit** | Beckman Coulter | A29154 |
| **DMEM High glucose** | Sigma Aldrich | D6429 |
| **DNA Isolation Kit** | Genfind | A41497 |
| **DNA Extraction Kit (Maxwell 16 Blood)** | Promega | AS1010 |
| **dNTP** | Invitrogen | 10297-117 |
| **EBNA-1 IgG ELISA Kit** | DiaSorin | P001607 |
| **ECL substrate** | BioRad | 170-5061 |
| **Ethylenediaminetetraacetic Acid** | Becton Dickinson | 367873 |
| **Ficoll** | VWR International | 17-1440-03 |
| **Foetal Bovine Serum (FBS)** | Scientific Partners (Serana) | S-FBS AU-015 |
| **Ham's Nutrient F12 Media** | Sigma Aldrich | 51651C |
| **magMAX Express 96 Deep Well Plate** | Life Technologies | 4388476 |
| **MgCl2** | Invitrogen | Y02016 |
| **Penicillin-Streptomycin-Glutamine (100X)** | Invitrogen | 10378-016 |
| **Phosphate Buffer Saline (PBS) Tablets** | Sigma Aldrich | P4417 |
| **Platinum Taq DNA polymerase** | Invitrogen | 10966034 |
| **Protein-interaction Pull-Down Kit** | ThermoFisher Scientific | 21115 |
| **Protran Supported 0.45um membrane** | GE Healthcare | 10600016 |
| **QIAamp DNA Extraction Kit** | Qiagen | 51106 |
| **RPMI 1640** | Invitrogen | 21870092 |

| | | |
|---|---|---|
| **SDS** | Astral Scientific Pty Ltd | 227 |
| **Secondary Antibody- IgG1** | Sapphire Biosciences | ab99774 |
| **Secondary Antibody- IgG2** | Sapphire Biosciences | ab99779 |
| **Secondary Antibody- IgG3** | Sapphire Biosciences | ab99829 |
| **Secondary Antibody- Total IgG** | Sigma Aldrich | A0170 |
| **Skim Milk powder** | Diploma | N/A |
| **Sodium Azide (Naxx)** | Sigma Aldrich | S2002 |
| **Sodium Chloride (NaCl)** | Sigma Aldrich | S7653 |
| **Serum Separating Tubes (SST)** | Becton Dickinson | 367958 |
| **Steritop filter unit** | Merck Millipore | SCGPT05RE |
| **Streptavidin-coated 96 well plates** | ThermoFisher Scientific | 436014 |
| **Taq (Expand High Fidelity for HLA)** | Roche | 11732641001 |
| **Tris (Trizma Base)** | Sigma Aldrich | T6066 |
| **Tris-Buffered Saline (TBS)** | ThermoFisher Scientific | 28376 |
| **Triton X100** | Sigma Aldrich | X100-1L |
| **Tween 20** | BDH | 663684B |
| **VCA IgG ELISA Kit** | DiaSorin | P001606 |
| **Viraclean** | Statewide Cleaning Supplies | 210556 |

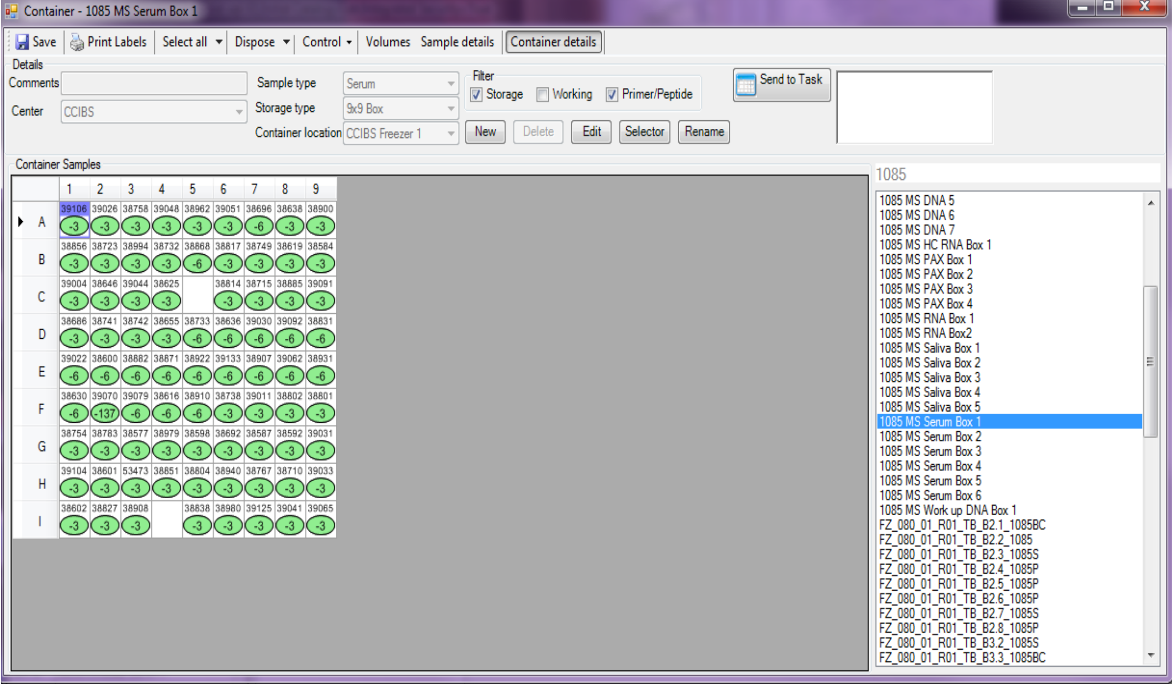
**Supplementary Table S-2. Abbreviations for amino acids.**

| Amino Acid | Three letter abbreviation | One letter symbol |
|---|---|---|
| **Alanine** | Ala | A |
| **Arginine** | Arg | R |
| **Asparagine** | Asn | N |
| **Aspartic acid** | Asp | D |
| **Asparagine or aspartic acid** | Asx | B |
| **Cysteine** | Cys | C |
| **Glutamine** | Gln | Q |
| **Glutamic acid** | Glu | E |
| **Glutamine or glutamic acid** | Glx | Z |
| **Glycine** | Gly | G |
| **Histidine** | His | H |
| **Isoleucine** | Ile | I |
| **Leucine** | Leu | L |
| **Lysine** | Lys | K |
| **Methionine** | Met | M |
| **Phenylalanine** | Phe | F |
| **Proline** | Pro | P |
| **Serine** | Ser | S |
| **Threonine** | Thr | T |
| **Tryptophan** | Trp | W |
| **Tyrosine** | Tyr | Y |
| **Valine** | Val | V |

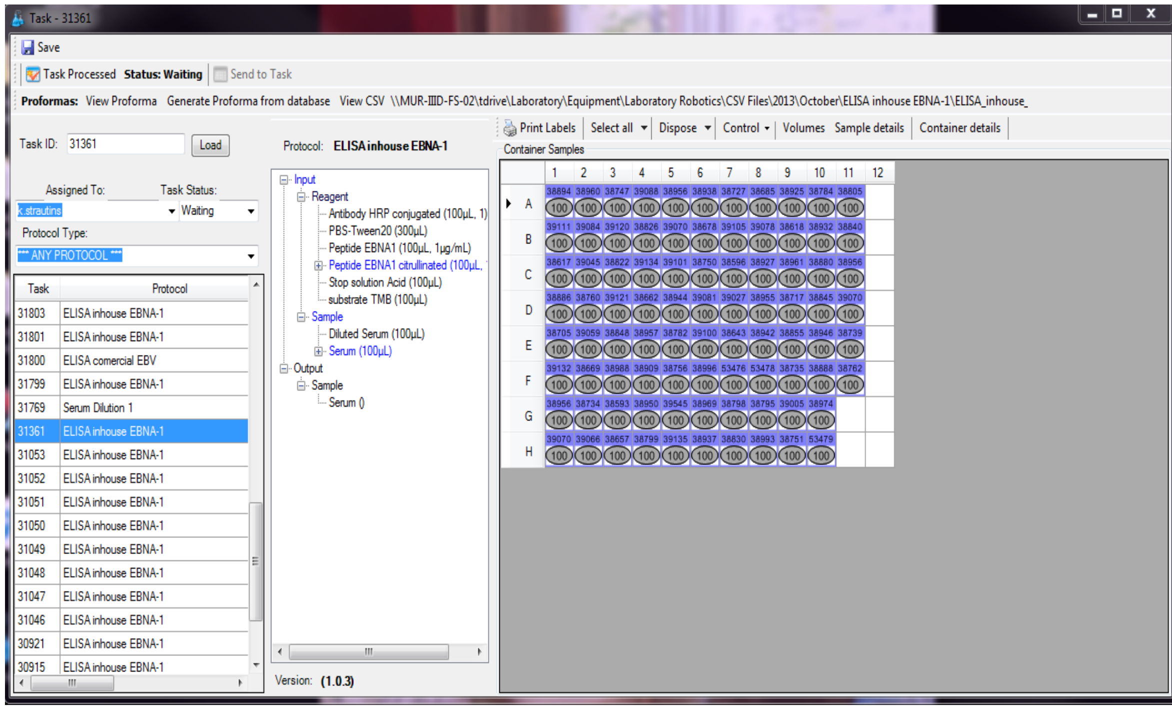



**Supplementary Figure S-1. Positive control dot blot (right) for macroarray (left).** Image is merged, as where white signal on dot blot is positive ECL signal. Serum was serially diluted neat, 1:2, 1:4, 1:8, 1:16, negative (no serum) and 1:32. This example shows that the positive dot blot worked, but no signal was seen on the macroarray.

A

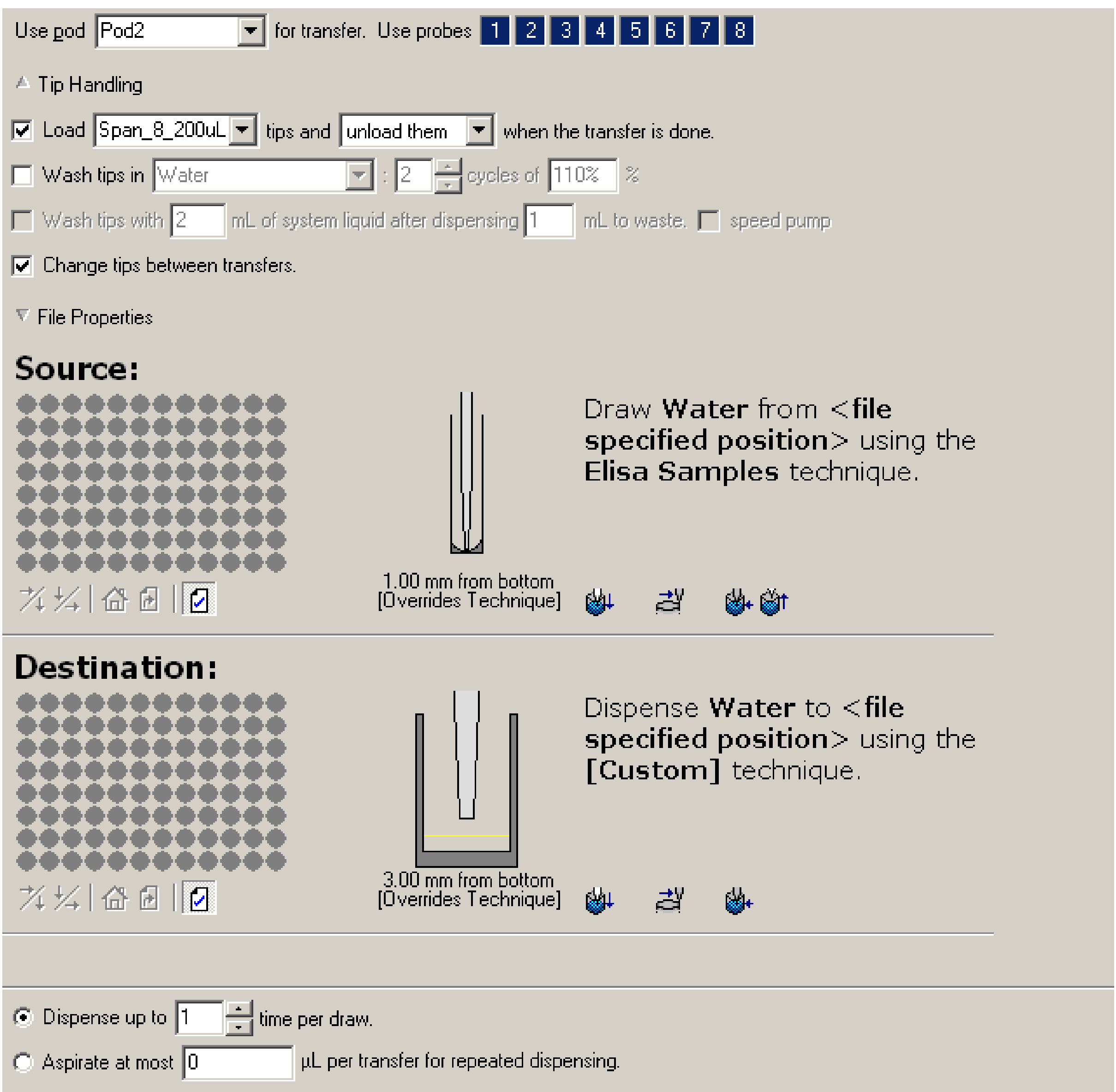



B




**Supplementary Figure S-2.** Layout of EpiLab sample tracking program, where samples from storage containers (A) can be tracked to ELISA protocols (B).

**Supplementary Figure S-3. ELISA protocol steps robot layout on BioMek.** Coating plates (A), sample addition (B) and secondary antibody addition (C) using appropriate reagent storage, pipette tips (dark green) and ELISA plates (dark red).




**Supplementary Figure S-4. BioMek FX$^{P}$ control of pipetting accuracy and precision.** Pressurized volume control for Span 8 (8 head pipette which can be control independent volumes. This can control depth of pipette tip into reservoir or plate, speed of aspiration and dispensation.

**Supplementary Table S-3. Raw values of serially-diluted internal control (IC) for commercial and in-house ELISAs.**

**i) EBNA-1**

| **Dilution** | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Plate 5 | Plate 6 | Plate 7 | Plate 8 | Plate 9 | Plate 10 | Plate 11 | Plate 12 | Plate 13 | Plate 14 | Plate 15 | Plate 16 | Plate 17 | Plate 18 | **Mean** | **StDev** | **%CV** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 280.0 | 0.847 | 0.861 | 0.836 | 0.814 | 0.826 | 0.800 | 0.840 | 0.816 | 0.751 | 0.775 | 0.791 | 0.880 | 0.779 | 0.813 | 0.807 | 0.855 | 0.892 | 0.813 | 0.822 | 0.037 | 4.490 |
| 392.0 | 0.640 | 0.628 | 0.671 | 0.629 | 0.592 | 0.597 | 0.660 | 0.631 | 0.559 | 0.581 | 0.612 | 0.624 | 0.610 | 0.654 | 0.601 | 0.649 | 0.607 | 0.574 | 0.618 | 0.031 | 4.990 |
| 548.8 | 0.466 | 0.489 | 0.492 | 0.485 | 0.498 | 0.461 | 0.474 | 0.507 | 0.416 | 0.459 | 0.508 | 0.481 | 0.449 | 0.497 | 0.476 | 0.497 | 0.486 | 0.442 | 0.477 | 0.024 | 5.089 |
| 768.3 | 0.330 | 0.367 | 0.398 | 0.407 | 0.375 | 0.363 | 0.383 | 0.387 | 0.337 | 0.349 | 0.384 | 0.381 | 0.368 | 0.384 | 0.398 | 0.389 | 0.383 | 0.331 | 0.373 | 0.023 | 6.152 |
| 1075.6 | 0.246 | 0.254 | 0.268 | 0.263 | 0.271 | 0.251 | 0.276 | 0.282 | 0.245 | 0.256 | 0.261 | 0.267 | 0.249 | 0.283 | 0.271 | 0.276 | 0.262 | 0.260 | 0.263 | 0.012 | 4.476 |
| 1505.9 | 0.190 | 0.209 | 0.203 | 0.191 | 0.193 | 0.184 | 0.196 | 0.199 | 0.168 | 0.181 | 0.186 | 0.180 | 0.193 | 0.200 | 0.191 | 0.184 | 0.196 | 0.182 | 0.190 | 0.010 | 5.070 |
| 2108.3 | 0.149 | 0.129 | 0.152 | 0.145 | 0.143 | 0.118 | 0.143 | 0.133 | 0.125 | 0.140 | 0.145 | 0.141 | 0.134 | 0.145 | 0.143 | 0.147 | 0.140 | 0.133 | 0.139 | 0.009 | 6.377 |

Stdev: Standard deviation. CV: Coefficient of Variation = (StDev/mean) x 100

**ii) VCA**

| **Dilution** | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Plate 5 | Plate 6 | Plate 7 | Plate 8 | Plate 9 | Plate 10 | Plate 11 | Plate 12 | Plate 13 | Plate 14 | Plate 15 | Plate 16 | Plate 17 | Plate 18 | **Mean** | **StDev** | **%CV** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 280.0 | 2.340 | 2.349 | 2.208 | 1.968 | 2.171 | 2.378 | 2.172 | 2.354 | 2.085 | 2.257 | 2.239 | 2.147 | 2.0184 | 2.227 | 2.236 | 2.163 | 2.352 | 2.521 | 2.232 | 0.137 | 6.115 |
| 392.0 | 1.874 | 1.917 | 1.839 | 1.665 | 1.659 | 1.906 | 1.822 | 1.987 | 1.690 | 2.043 | 1.892 | 1.684 | 1.8375 | 1.770 | 1.785 | 1.791 | 1.865 | 1.646 | 1.815 | 0.115 | 6.339 |
| 548.8 | 1.636 | 1.561 | 1.584 | 1.466 | 1.548 | 1.737 | 1.575 | 1.708 | 1.522 | 1.685 | 1.538 | 1.468 | 1.5456 | 1.602 | 1.559 | 1.471 | 1.651 | 1.364 | 1.568 | 0.094 | 5.992 |
| 768.3 | 1.298 | 1.286 | 1.321 | 1.085 | 1.301 | 1.433 | 1.259 | 1.461 | 1.254 | 1.422 | 1.309 | 1.206 | 1.3568 | 1.349 | 1.287 | 1.238 | 1.374 | 1.129 | 1.298 | 0.098 | 7.534 |
| 1075.6 | 1.054 | 0.931 | 0.929 | 0.808 | 0.889 | 0.994 | 0.949 | 1.025 | 0.872 | 0.925 | 0.966 | 0.855 | 0.9161 | 0.946 | 0.913 | 0.894 | 0.994 | 1.030 | 0.938 | 0.064 | 6.858 |
| 1505.9 | 0.780 | 0.720 | 0.706 | 0.628 | 0.679 | 0.794 | 0.712 | 0.778 | 0.620 | 0.754 | 0.758 | 0.657 | 0.7274 | 0.765 | 0.688 | 0.641 | 0.764 | 0.676 | 0.714 | 0.055 | 7.741 |
| 2108.3 | 0.597 | 0.544 | 0.563 | 0.508 | 0.506 | 0.594 | 0.536 | 0.585 | 0.555 | 0.548 | 0.538 | 0.511 | 0.5578 | 0.559 | 0.530 | 0.505 | 0.574 | 0.563 | 0.549 | 0.029 | 5.308 |

Grey plates indicate experiments for which data was used in analysis (Chapter 4)

### iii) EBNA-1$_{(398-413)}$

| Dilution | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Plate 5 | Plate 6 | Plate 7 | Plate 8 | Plate 9 | Plate 10 | Plate 11 | Plate 12 | Plate 13 | Plate 14 | Plate 15 | Plate 16 | Plate 17 | Plate 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 768.3 | 1.478 | 1.637 | 0.632 | 0.726 | 1.169 | 1.287 | 1.610 | 1.394 | 0.880 | 1.138 | 1.440 | 0.974 | 0.796 | 0.885 | 0.881 | 1.001 | 1.341 | 0.959 |
| 1075.6 | 1.103 | 0.950 | 0.500 | 0.545 | 0.871 | 1.085 | 1.210 | 0.753 | 0.793 | 0.768 | 1.000 | 0.870 | 0.624 | 0.702 | 0.810 | 0.969 | 0.898 | 0.977 |
| 1505.9 | 0.805 | 0.928 | 0.375 | 0.354 | 0.620 | 0.665 | 0.733 | 0.866 | 0.614 | 0.786 | 0.770 | 0.539 | 0.385 | 0.520 | 0.652 | 0.771 | 0.699 | 0.692 |
| 2108.3 | 0.532 | 0.611 | 0.347 | 0.302 | 0.557 | 0.540 | 0.509 | 0.487 | 0.411 | 0.569 | 0.592 | 0.595 | 0.418 | 0.395 | 0.488 | 0.584 | 0.570 | 0.539 |
| 2951.6 | 0.368 | 0.318 | 0.201 | 0.201 | 0.358 | 0.322 | 0.355 | 0.335 | 0.400 | 0.308 | 0.339 | 0.308 | 0.345 | 0.266 | 0.345 | 0.433 | 0.446 | 0.419 |
| 4132.2 | 0.212 | 0.243 | 0.161 | 0.143 | 0.260 | 0.248 | 0.238 | 0.235 | 0.223 | 0.228 | 0.327 | 0.223 | 0.171 | 0.262 | 0.172 | 0.301 | 0.322 | 0.316 |
| 5785.1 | 0.189 | 0.170 | 0.084 | 0.098 | 0.186 | 0.144 | 0.173 | 0.176 | 0.221 | 0.161 | 0.241 | 0.150 | 0.184 | 0.211 | 0.198 | 0.167 | 0.236 | 0.229 |

| Dilution | Plate 19 | Plate 20 | Plate 21 | Plate 22 | Plate 23 | Plate 24 | Plate 25 | Plate 26 | Plate 27 | Plate 28 | Plate 29 | Plate 30 | Plate 31 | Plate 32 | Plate 33 | Plate 34 | Plate 35 | Plate 36 | **Mean** | **StDev** | **%CV** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 768.3 | 0.949 | 0.980 | 0.799 | 0.795 | 0.842 | 0.902 | 1.370 | 1.377 | 1.277 | 1.054 | 0.916 | 1.379 | 1.106 | 1.140 | 1.155 | 0.846 | 0.750 | 0.766 | 1.073 | 0.269 | 25.06 |
| 1075.6 | 0.995 | 1.056 | 0.600 | 0.597 | 0.636 | 0.640 | 0.646 | 1.027 | 0.993 | 0.651 | 0.892 | 0.988 | 0.960 | 0.808 | 0.920 | 0.789 | 0.755 | 0.552 | 0.831 | 0.182 | 21.94 |
| 1505.9 | 0.504 | 0.604 | 0.480 | 0.433 | 0.472 | 0.578 | 0.496 | 0.797 | 0.599 | 0.479 | 0.729 | 0.852 | 0.853 | 0.571 | 0.611 | 0.555 | 0.463 | 0.421 | 0.619 | 0.155 | 25.08 |
| 2108.3 | 0.422 | 0.409 | 0.487 | 0.324 | 0.481 | 0.431 | 0.598 | 0.553 | 0.586 | 0.547 | 0.516 | 0.503 | 0.610 | 0.449 | 0.351 | 0.821 | 0.408 | 0.318 | 0.496 | 0.107 | 21.47 |
| 2951.6 | 0.419 | 0.335 | 0.318 | 0.254 | 0.252 | 0.302 | 0.503 | 0.311 | 0.318 | 0.496 | 0.391 | 0.430 | 0.355 | 0.377 | 0.417 | 0.196 | 0.233 | 0.340 | 0.342 | 0.076 | 22.34 |
| 4132.2 | 0.215 | 0.236 | 0.204 | 0.176 | 0.225 | 0.220 | 0.330 | 0.339 | 0.316 | 0.348 | 0.281 | 0.312 | 0.240 | 0.250 | 0.282 | 0.156 | 0.231 | 0.240 | 0.247 | 0.055 | 22.45 |
| 5785.1 | 0.145 | 0.172 | 0.146 | 0.115 | 0.170 | 0.139 | 0.239 | 0.242 | 0.139 | 0.255 | 0.209 | 0.222 | 0.247 | 0.192 | 0.205 | 0.135 | 0.124 | 0.186 | 0.181 | 0.044 | 24.35 |
| Blank | 0.030 | 0.032 | 0.034 | 0.033 | 0.031 | 0.031 | 0.029 | 0.032 | 0.034 | 0.029 | 0.030 | 0.030 | 0.032 | 0.033 | 0.029 | 0.032 | 0.031 | 0.032 | 0.031 | 0.002 | 7.37 |

Grey plates indicate experiments for which data was used in analysis (Chapter 4)

**iv) EBNA-1$_{(398-413\ CIT)}$**

| Dilution | Plate 1 | Plate 2 | Plate 3 | Plate 4 | Plate 5 | Plate 6 | Plate 7 | Plate 8 | Plate 9 | Plate 10 | Plate 11 | Plate 12 | Plate 13 | Plate 14 | Plate 15 | Plate 16 | Plate 17 | Plate 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 768.3 | 0.814 | 0.850 | 0.587 | 0.819 | 0.902 | 0.931 | 1.219 | 0.875 | 0.801 | 0.779 | 1.015 | 1.009 | 0.631 | 1.014 | 0.916 | 0.754 | 0.741 | 1.267 |
| 1075.6 | 0.677 | 0.611 | 0.405 | 0.473 | 0.694 | 0.630 | 0.662 | 0.673 | 0.687 | 0.564 | 0.773 | 0.756 | 0.690 | 0.500 | 0.611 | 0.536 | 0.908 | 0.688 |
| 1505.9 | 0.541 | 0.451 | 0.335 | 0.236 | 0.520 | 0.611 | 0.622 | 0.614 | 0.423 | 0.424 | 0.466 | 0.456 | 0.537 | 0.392 | 0.537 | 0.528 | 0.415 | 0.688 |
| 2108.3 | 0.359 | 0.328 | 0.230 | 0.312 | 0.387 | 0.327 | 0.362 | 0.356 | 0.374 | 0.372 | 0.329 | 0.309 | 0.358 | 0.277 | 0.224 | 0.468 | 0.296 | 0.348 |
| 2951.6 | 0.273 | 0.210 | 0.130 | 0.210 | 0.337 | 0.248 | 0.354 | 0.317 | 0.297 | 0.224 | 0.256 | 0.335 | 0.151 | 0.203 | 0.195 | 0.341 | 0.198 | 0.353 |
| 4132.2 | 0.186 | 0.140 | 0.102 | 0.164 | 0.205 | 0.161 | 0.175 | 0.168 | 0.230 | 0.162 | 0.240 | 0.151 | 0.129 | 0.139 | 0.099 | 0.128 | 0.149 | 0.198 |
| 5785.1 | 0.137 | 0.106 | 0.073 | 0.083 | 0.125 | 0.122 | 0.117 | 0.172 | 0.122 | 0.130 | 0.187 | 0.112 | 0.122 | 0.115 | 0.115 | 0.104 | 0.096 | 0.177 |

| Dilution | Plate 19 | Plate 20 | Plate 21 | Plate 22 | Plate 23 | Plate 24 | Plate 25 | Plate 26 | Plate 27 | Plate 28 | Plate 29 | Plate 30 | Plate 31 | Plate 32 | Plate 33 | Plate 34 | Plate 35 | Plate 36 | Mean | StDev | %CV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 768.3 | 0.920 | 0.737 | 1.050 | 0.684 | 0.696 | 0.719 | 1.284 | 1.281 | 1.372 | 0.923 | 1.115 | 1.190 | 1.206 | 0.913 | 1.045 | 0.724 | 0.712 | 0.925 | 0.928 | 0.207 | 22.33 |
| 1075.6 | 0.778 | 0.556 | 0.558 | 0.784 | 0.547 | 0.549 | 0.989 | 0.683 | 0.928 | 0.636 | 0.458 | 0.879 | 0.825 | 0.616 | 0.675 | 0.469 | 0.552 | 0.720 | 0.659 | 0.138 | 20.98 |
| 1505.9 | 0.704 | 0.370 | 0.390 | 0.478 | 0.415 | 0.429 | 0.631 | 0.677 | 0.776 | 0.680 | 0.674 | 0.630 | 0.589 | 0.415 | 0.334 | 0.309 | 0.375 | 0.506 | 0.505 | 0.131 | 25.85 |
| 2108.3 | 0.352 | 0.298 | 0.394 | 0.303 | 0.304 | 0.369 | 0.358 | 0.538 | 0.381 | 0.320 | 0.318 | 0.460 | 0.328 | 0.324 | 0.451 | 0.557 | 0.256 | 0.273 | 0.350 | 0.073 | 20.96 |
| 2951.6 | 0.241 | 0.191 | 0.250 | 0.280 | 0.191 | 0.207 | 0.212 | 0.242 | 0.264 | 0.387 | 0.311 | 0.348 | 0.260 | 0.235 | 0.220 | 0.158 | 0.208 | 0.185 | 0.251 | 0.065 | 25.84 |
| 4132.2 | 0.229 | 0.143 | 0.159 | 0.203 | 0.148 | 0.147 | 0.137 | 0.285 | 0.236 | 0.247 | 0.193 | 0.160 | 0.174 | 0.155 | 0.162 | 0.100 | 0.125 | 0.134 | 0.168 | 0.044 | 25.93 |
| 5785.1 | 0.187 | 0.106 | 0.117 | 0.115 | 0.104 | 0.124 | 0.184 | 0.191 | 0.202 | 0.206 | 0.116 | 0.158 | 0.140 | 0.114 | 0.095 | 0.074 | 0.102 | 0.100 | 0.129 | 0.036 | 28.10 |

Grey plates indicate experiments for which data was used in analysis (Chapter 5)

## v) EBNA-1$_{(398-413)}$ IgG Subclasses

| | **1** | | | **2** | | | **3** | | | **4** | | | **5** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilution** | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 |
| 768.32 | 0.833 | 0.995 | 0.982 | 0.715 | 0.732 | 0.863 | 0.672 | 0.652 | 1.012 | 0.970 | 0.724 | 1.038 | 0.708 | 0.952 | 0.698 |
| 1075.648 | 0.580 | 0.726 | 0.664 | 0.454 | 0.508 | 0.452 | 0.483 | 0.498 | 0.659 | 0.680 | 0.574 | 0.733 | 0.589 | 0.615 | 0.517 |
| 1505.907 | 0.529 | 0.483 | 0.508 | 0.337 | 0.362 | 0.315 | 0.339 | 0.390 | 0.496 | 0.580 | 0.436 | 0.487 | 0.334 | 0.449 | 0.446 |
| 2108.27 | 0.323 | 0.340 | 0.406 | 0.241 | 0.280 | 0.255 | 0.270 | 0.260 | 0.373 | 0.399 | 0.331 | 0.342 | 0.319 | 0.341 | 0.283 |
| 2951.578 | 0.229 | 0.261 | 0.259 | 0.186 | 0.212 | 0.192 | 0.194 | 0.182 | 0.204 | 0.317 | 0.239 | 0.259 | 0.268 | 0.248 | 0.211 |
| 4132.209 | 0.224 | 0.249 | 0.273 | 0.142 | 0.158 | 0.137 | 0.145 | 0.144 | 0.206 | 0.236 | 0.190 | 0.199 | 0.182 | 0.183 | 0.162 |
| 5785.093 | 0.215 | 0.174 | 0.199 | 0.116 | 0.125 | 0.160 | 0.119 | 0.153 | 0.133 | 0.184 | 0.155 | 0.155 | 0.153 | 0.141 | 0.133 |

| | **6** | | | 7 | | | 8 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dilution** | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 | IgG1 | IgG2 | IgG3 | **Average** | **StDev** | **%CV** |
| 768.32 | 1.068 | 0.941 | 1.079 | 0.894 | 1.001 | 0.881 | 0.578 | 0.784 | 0.624 | 0.869 | 0.151 | 17.4 |
| 1075.648 | 0.818 | 0.638 | 0.796 | 0.651 | 0.830 | 0.662 | 0.418 | 0.591 | 0.474 | 0.610 | 0.113 | 18.5 |
| 1505.907 | 0.497 | 0.474 | 0.548 | 0.403 | 0.667 | 0.442 | 0.327 | 0.338 | 0.310 | 0.445 | 0.081 | 18.2 |
| 2108.27 | 0.384 | 0.347 | 0.424 | 0.326 | 0.479 | 0.340 | 0.264 | 0.208 | 0.284 | 0.329 | 0.055 | 16.8 |
| 2951.578 | 0.282 | 0.263 | 0.316 | 0.261 | 0.380 | 0.292 | 0.160 | 0.210 | 0.220 | 0.240 | 0.042 | 17.5 |
| 4132.209 | 0.241 | 0.213 | 0.238 | 0.197 | 0.234 | 0.266 | 0.125 | 0.118 | 0.178 | 0.196 | 0.042 | 21.5 |
| 5785.093 | 0.234 | 0.167 | 0.183 | 0.149 | 0.197 | 0.226 | 0.096 | 0.100 | 0.113 | 0.161 | 0.033 | 20.3 |

**Supplementary Table S-4. Full names and codes of proteins from HexSelect macroarray.**

| Protein Code | Protein Name |
|---|---|
| STMN4 | Stathmin-4 |
| HDAC5 | Histone deacetylase 5 |
| MBD3 | Methyl-CpG binding domain protein 3 |
| PIM3 | Serine/threonin-protein kinase Pim-3 |
| EPN1 | Epsin-1 |
| JMJD8 | Jumonji domain-containing protein 8 |
| SHB2 | SH2 domain-containing adapter protein B |
| HBA2 | Haemoglobin subunit alpha |
| EEF1A2 | Elongation factor 1-alpha 2 (Statin S1) |
| TROVE2 | 60 kDa SS-A/Ro ribonucleoprotein |
| NFKBIL2 | NF-kappa B inhibitor like protein 2 |
| KAT2A | Histone acetyltransferase |
| CENPB | Major centromere autoantigen B |
| AMPD2 | AMP deaminase 2 |
| MAP1LC3A | Microtubule-associated protein 1A/1B light chain 3A precursor |
| UFC1 | Ubiquitin-fold modifier-conjugating enzyme 1 |
| AZGP1 | Zinc-alpha-2-glycoprotein |

**Supplementary Table S-5. Raw data of cell viability counts of MO3.13 and SH-SY5Y with MS serum.**

| | | MO3.13 | | | SH-SY5Y | | |
|---|---|---|---|---|---|---|---|
| **Time point** | **Conditions** | **Alive** | **Dead** | **% Viability** | **Alive** | **Dead** | **% Viability** |
| **6 hours** | No serum | 45 | 2 | 95.7 | 5 | 0 | 100.0 |
| | | 42 | 4 | 91.3 | 13 | 1 | 92.9 |
| | | 39 | 0 | 100.0 | 10 | 0 | 100.0 |
| | serum 1/100 | 21 | 1 | 95.5 | 28 | 2 | 93.3 |
| | | 39 | 1 | 97.5 | 33 | 0 | 100.0 |
| | | 37 | 3 | 92.5 | 9 | 0 | 100.0 |
| | serum 1/1000 | 10 | 4 | 71.4 | 20 | 5 | 80.0 |
| | | 17 | 2 | 89.5 | 27 | 0 | 100.0 |
| | | 61 | 5 | 92.4 | 28 | 11 | 71.8 |
| **24 hours** | No serum | 58 | 4 | 93.5 | 101 | 2 | 98.1 |
| | | 39 | 1 | 97.5 | 45 | 3 | 93.8 |
| | | 80 | 3 | 96.4 | 62 | 1 | 98.4 |
| | serum 1/100 | 38 | 1 | 97.4 | 48 | 2 | 96.0 |
| | | 60 | 3 | 95.2 | 41 | 0 | 100.0 |
| | | 66 | 4 | 94.3 | 45 | 2 | 95.7 |
| | serum 1/1000 | 17 | 3 | 85.0 | 27 | 11 | 71.1 |
| | | 99 | 3 | 97.1 | 30 | 2 | 93.8 |
| | | 77 | 4 | 95.1 | 24 | 5 | 82.8 |
| **48 hours** | No serum | 60 | 4 | 93.8 | 11 | 7 | 61.1 |
| | | 52 | 3 | 94.5 | 23 | 4 | 85.2 |
| | | 66 | 6 | 91.7 | 23 | 4 | 85.2 |
| | serum 1/100 | 61 | 4 | 93.8 | 42 | 12 | 77.8 |
| | | 71 | 7 | 91.0 | 36 | 6 | 85.7 |
| | | 62 | 10 | 86.1 | 25 | 12 | 67.6 |
| | serum 1/1000 | 23 | 4 | 85.2 | 31 | 2 | 93.9 |
| | | 66 | 5 | 93.0 | 22 | 4 | 84.6 |
| | | 88 | 10 | 89.8 | 40 | 8 | 83.3 |

1/100: dilution of serum in total cell media volume.