NEUTRALIZING ANTIBODIES AND THE BIOLOGICAL
RESPONSE TO INTERFERON-BETA THERAPY

by

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Abstract

Interferon-beta (IFN-beta) was the first disease modifying drug to be approved for the treatment of multiple sclerosis (MS). IFN-beta reduces relapse rates, relapse severity as well as slows the progression of disease burden. However, neutralizing antibodies (NAbs) occur in a proportion of patients receiving IFN-beta treatment. NAbs bind to IFN-beta, reduce drug bioavailability and high levels of NAbs reduce drug efficiency.

Our first objective was to develop and validate a sensitive and rapid method to measure NAbs. Existing methods to measure NAbs include the cytopathic effect assay (CPE) method and the myxovirus protein A (MxA) assay. However, these assays are time consuming and may be arduous to perform. We describe the optimization of a luciferase reporter gene assay to measure NAbs. To validate the assay, we assayed sera from IFN-beta treated MS patients with the optimized luciferase method and compared the results to those obtained with the reference CPE method. NAb status measured by the luciferase and the CPE method did not yield a significant difference. NAb titres obtained from the two methods correlated very well. The luciferase assay is reliable, appropriately sensitive and requires less time than the currently available NAb methods.

In addition to measuring NAbs, the biological activity of IFN-beta can be measured by monitoring IFN-inducible biomarkers, specifically MxA mRNA. Bioavailability measurements become especially valuable in patients with low to moderate NAb titres, “the grey zone”, and have been identified as a possible alternative for NAb measurements. Nonetheless, there is still controversy about how long should one wait after an IFN injection to draw blood for MxA mRNA measurements. Our objective was to identify the optimal time for blood draws to measure MxA mRNA. MxA mRNA was the most robust at 4-12 hours after IFN-beta injection and peaked at 8h post IFN injection. NAbs were evidently associated with an attenuation of IFN-beta bioactivity.

In conclusion, we characterized a technique to assess NAbs associated with IFN-beta therapy and a method to assess IFN-beta biological activity in treated patients. Altogether, this will help measure the effects of IFN-beta treatment and assist clinicians in tailoring therapy to the individual patient.
Preface

Publications


Co-authorship Statements

The research which appears in Chapter 2 of this thesis was designed by R. Lam. R. Lam validated parameters that optimize assay sensitivity and reproducibility to improve the R. Farrell and G. Giovannoni luciferase assay methodology for the detection of NAbs to IFN-beta. NAb testing by luciferase was done by R. Lam. BAb testing by ELISA was done by E. Gibbs. NAb testing by CPE was done by S. Grossberg. R. Lam analysed the data. All co-authors contributed to the revision of the manuscript which was written and prepared by R. Lam for submission.

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Introduction
1.1 Multiple sclerosis the disease

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammatory demyelinating lesions in the central nervous system (CNS). MS is generally regarded as a T cell autoimmune disease, where T cells recognizing myelin or oligodendrocyte antigens cross the blood brain barrier (BBB) to cause inflammation in the CNS. In addition to acute inflammation, a degenerative process also occurs and contributes to disability progression (reviewed by McFarland and Martin, 2007). Symptoms for the disease involve reduced vision, motor weakness, sensory discomfort, impaired coordination and balance, as well as bowel, bladder and sexual dysfunction, and cognitive difficulties (reviewed by Compston, et al., 2002; Noseworthy, et al., 2000). MS is the most common neurodegenerative disorder with onset in young adults. Canada has a prevalence of at least 100 per 100 000, which is among the highest in the world (Poppe, et al., 2008; World Health Organization, 2008).

1.2 MS disease etiology

The exact cause of multiple sclerosis remains unknown. A combination of environmental factors and infectious disease likely contributes to MS in genetically susceptible individuals.

1.2.1 Genetic factors

MS twin studies have shown that there is a genetic basis to MS (Hafler, 2004). MS was the first autoimmune disease to be associated with the human leukocyte antigen (HLA). In the 1970's, the major histocompatibility complex (MHC) class I molecules HLA-A3 (Naito, et al., 1972) HLA-A3 and -B7 (Jersild, et al., 1972), as well as HLA-A3, -A10, and -B35 (Bertrams, et al., 1972) were found to be associated with MS, these findings were subsequently attributed to the linked HLA-DR2 alleles in the MHC class II region. Several other genes have also been identified for MS disease and confirmed in different datasets, including the interleukin (IL)-7 receptor α gene (IL7RA), the IL-2 receptor α gene (IL2RA), as well as CD58, CD226, CLEC16A, EVI5 and TYK2 (Gregory, et al., 2007; ANZgene, 2009; Hoppenbrouwers, et al., 2009; IMSGC, 2009; Zoledziewska, et al., 2009). The HLA associations seem to have the strongest genetic effect; genetic effects of other genes are more moderate in size (GAMES, 2003; Sawcer, et al., 2005).
1.2.2 Environmental factors

1.2.2.1 Non-infectious environment factors

Cigarette smoking and sun exposure are some of the environmental factors that may contribute to MS.

1.2.2.1.1 Cigarette smoking

Tobacco smoking has been associated with the risk of developing MS (Hawkes, 2007; Sundström, et al., 2008; Hedström, et al., 2009), disease progression in patients (Di Pauli, et al., 2008) and worsening the prognosis of MS (Sundström, et al., 2008; Healy, et al., 2009). Nevertheless, this is not a consistent observation as others have found no significant association of cigarette smoking and MS in multiplex families with shared genetic-environmental risk factors (Jafari, et al., 2009). The increased risk of MS among smokers can be explained by several hypotheses. The increase of nitric oxide production, the higher frequency of respiratory infections in addition to the neurotoxic effects of cyanides and other components of cigarette smoke may have effects on the immune system and vascular system (Hernán, et al., 2001; Hernán, et al., 2005). Furthermore, nicotine and other components of cigarette smoke could alter blood brain barrier function and modify endothelial cell function (Hawkins, et al., 2002).

1.2.2.1.2 Vitamin D and sun exposure

MS incidence and prevalence increase with latitude both north and south of the equator (Kurtzke, et al., 1985; McLeod, et al., 1994); low dietary vitamin D intake and low serum levels of the vitamin D metabolite 25-hydroxyvitamin D is associated with an increased risk of developing MS (Munger, et al., 2004; Munger, et al., 2006). As the most important source of vitamin D is from its synthesis in the skin when exposed to sunlight (ultraviolet B radiation), sun exposure and Vitamin D are attractive candidates to explain environmental factors of MS development (Raghuwanshi, et al., 2008; MacLean and Freedman, 2009; Myhr, 2009). 25-hydroxyvitamin D can mediate anti-inflammatory processes by reducing MHC-class II, co-stimulatory molecules and pro-inflammatory cytokine expression in monocytes and antigen presenting cells (APCs). This can reduce T-cell proliferation and induce the apoptosis of activated T cells. 1,25-dihydroxyvitamin D can inhibit B cell proliferation and induce B cell apoptosis, reducing immunoglobulin production (reviewed by Smolders, et al., 2008). Intervention with vitamin D supplement is safe and well tolerated in treated individuals. Vitamin D treated patients experienced fewer relapses than control groups in several studies although this needs to be verified in further trials as these studies lacked statistical precision to adequately
assess changes in clinical disease measures. (Kimball, et al., 2007; Burton, et al., 2008; Burton, et al., 2010).

1.2.2.2 Infectious environmental factors
Human herpes virus 6 (HHV6), Human endogenous retroviruses (HERVs) and Epstein-Barr virus (EBV) have been implicated in the development of multiple sclerosis. The center of attention is currently focused on EBV.

1.2.2.2.1 Epstein-Barr virus
EBV is one of the most common viruses in humans and most people become infected with EBV at some point during their lives. The virus infects resting B cells and immortalizes them into long-lived memory B cells which largely remain undetected by the immune system.

EBV in childhood often do not show symptoms or show only mild symptoms similar to other childhood illnesses. If the infection occurs in adolescence or young adulthood, infectious mononucleosis may occur but most individuals remain healthy.

Nearly all adult MS patients have evidence for prior EBV infection compared to 90-95% of all humans (reviewed by Bagert, 2009); children with MS have a higher seroprevalence of EBV compared to matched controls (Pohl, et al., 2006; Banwell, et al., 2007). These studies suggest that prior infection with EBV may be necessary for the development of MS (reviewed by Bagert, 2009). Evidence of dysregulated EBV and B cell follicle-like lymphoid aggregates was described in post-mortem MS brain tissue (Serafini, et al., 2007); however, others have reported that EBV infection was rare in the MS brain (Willis, et al., 2009).

EBV could potentially contribute to MS through molecular mimicry with myelin antigens. Epstein Barr Virus nuclear antigen 1 (EBNA-1) specific T cells from MS patients can cross-react with myelin antigens and co-produce inflammatory cytokines interferon (IFN)-gamma and IL-2 (Lunemann, et al., 2008).

1.2.3 Other factors

1.2.3.1 Chronic cerebrospinal venous insufficiency (CCSVI)
Recently, impaired venous function was described as a contributing factor in MS and has generated much excitement and media attention. Zamboni and colleagues observed that there were abnormalities of cerebral venous outflow in MS patients. The group used non-invasive echoclour Doppler (ECD) and transcranial colourcoded Doppler sonography (TCCS) to
investigate the haemodynamics of cerebral venous return. The group defined chronic cerebrospinal venous insufficiency (CCSVI) based on the presence of two of the following five parameters: 1) reflux in the internal jugular vein (IJV) and/or vertebral veins (VVs) in sitting and supine posture; 2) reflux in the deep cerebral veins (DCVs); 3) high-resolution B-mode evidence of IJV stenoses; 4) flow not Doppler-detectable in the IJVs and/or VVs; 5) reverted postural control of the main cerebral venous outflow pathways (Zamboni, et al., 2009a). The group studied 65 patients with clinically defined MS (CDMS) and 235 controls comprised of healthy subjects, healthy subjects older than CDMS patients, as well as patients with other neurological diseases and older controls not affected by neurological diseases with other pathologies. CDMS was strongly associated with CCSVI whereas controls did not show the presence of multiple sever extracranial stenosis affecting the principal cerebrospinal venous segments seen in MS. Relapsing-remitting multiple sclerosis (RRMS) and secondary progressive multiple sclerosis (SPMS) courses had CCSVI patterns different from PPMS.

The group later showed that percutaneous transluminal angioplasty (PTA) of venous strictures in patients with CCSVI was safe and could positively influence the postoperative clinical measures and quality of life parameters of MS in a prospective open-label study (Zamboni, et al., 2009b). However, a major shortcoming of this pilot study is that this was not a blinded study and bias could have played a role in the positive findings. The authors warrant a multicenter randomized controlled trial with blinded assessors of neurologic outcome to study the endovascular treatment of the associated CCSVI added to pharmacological treatment versus (vs.) pharmacological treatment alone.

1.3 Immunopathology of MS

MS is seen as an immune-mediated disease. Apart from demyelination, the other hallmark of the disease is inflammation. Current beliefs of key immunopathogenic processes in MS are based on studying the animal model of MS, experimental autoimmune encephalomyelitis (EAE) in mice.

1.3.1 Key immunopathogenic processes in MS

1.3.1.1 T cell activation and differentiation

The first event of the immunopathogenic process in MS is likely the activation of myelin-specific T cells in the periphery which occurs when the T cell receptor (TCR) of a naïve T cell encounters a myelin-like antigen presented by MHCII on the surface of antigen presenting cells.
(APCs). The interaction between costimulatory molecules on APCs (CD80, CD86, and CD40) and the T cell (CD28, CD40L) provides a second signal to continue T cell activation and differentiation into activated T helper cells, producing a pro-inflammatory immune response. The origin of the myelin-like antigen here is unknown; it may be the result of molecular mimicry of infectious organisms.

1.3.1.2 T cell proliferation
Activated T-helper cells release pro-inflammatory cytokines such as IL-2, IL-12, IFN-gamma and tumour necrosis factor (TNF)-alpha. IL-2 induces T cell proliferation and IL-12 is involved in the differentiation of naive T cells (Th0) into cells which will further develop into activated T helper (Th) cells. There are three subsets of T helper cells: Th1, Th2 and Th17. Th2 cells are involved with humoral immunity and are not generally associated with the initiation of MS immunopathogenesis. Both Th1 and the novel Th17 subset of T helper cells are capable of inducing and promoting multiple sclerosis (Juszczak and Głabiński, 2009).

1.3.1.3 Activated T Helper cells traffic across the blood brain barrier
Chemokines attract activated T cells towards the vascular endothelium. Upregulated adhesion molecules (integrin alpha4beta1 or VLA-4 or Very late antigen-4 or CD29/CD49d, CD6, integrin alphaLbeta2 or LFA-1or lymphocyte function-associated antigen 1) expressed on activated T cells and leukocytes bind to their cognate receptors (vascular cell adhesion molecule or VCAM-1, activated leukocyte cell adhesion molecule or ALCAM or CD166, Inter-Cellular Adhesion Molecule 1 or ICAM-1 or CD54, PECAM-1 or CD31) on the vascular endothelium (Greenwood, et al., 2002; Dietrich, 2002; Kalinowska and Losy, 2006; Cayrol, et al., 2008) to extravasate through the blood-brain barrier. Matrix metalloproteinases (MMPs) are secreted by activated T cells to degrade the extracellular matrix to facilitate migration of T cells into the CNS.

1.3.1.4 T cell reactivation and induction of immune cell-mediated demyelination
In the CNS, T cells are reactivated by APCs presenting CNS antigens. Activated T cells release pro-inflammatory cytokines, recruit innate immune cells, T cells, B cells, and macrophages and induce demyelination and axonal damage through TNF-alpha, reactive oxygen intermediates, nitric oxide intermediates, anti-myelin antibodies, and complement activation. Under the pro-inflammatory environment, perivascular cells and brain microvascular endothelial cells produce inflammatory factors which further affect the permeability of the blood brain barrier and stimulate the expression of adhesion molecules (reviewed by Dietrich, 2004).
1.3.2 Pathogenic immune cells in MS

Th1 CD4+ cells are well recognized in the pathogenesis of MS, but Th17 CD4+ cells, Th2 CD4+ cells, B cells, CD8+ T cells and T regulatory cells and also play a role.

1.3.2.1 Th1 cells

T helper 1 cells differentiate in the presence of IL-12 and secrete cytokines IFN-gamma and TNF-alpha to activate macrophages and cytotoxic T cells in order to achieve cell mediated immunity. Early studies of the autoimmunity in multiple sclerosis focused on the Th1 subset of T helper cells because many lines of evidence supported that they are the pro-inflammatory effectors of autoimmunity. In EAE susceptible mice, an animal model of MS, IFN-gamma levels in the CNS correlate with disease severity and levels of the cytokine peaked at the height of disease and fell when the disease was in remission (Merrill, et al., 1992). The source of IFN-gamma was from infiltrating CD4+ T cells (Renno, et al., 1995), and adoptive transfer of IFN-gamma producing T cell lines can induce EAE in naïve animals (Pettinelli, et al., 1981). Mice lacking Th1 lineage-specific transcription factors T-bet and STAT4 (signal transducer and activator of transcription 4) were protected from EAE (Bettelli, et al., 2004; Chitnis, et al., 2001). Administration of IFN-gamma to human MS patients exacerbates disease (Panitch, 1992). Mice lacking the IL-12p40 subunit were protected from EAE (Becher, et al., 2002). On the other hand, contradicting evidence is also present. Neither targeting IFN-gamma by genetic deletion nor by neutralizing antibodies confers resistance to EAE (Ferber, et al., 1996; Billiau, et al., 1988; Heremans, et al., 1996). Moreover, mice lacking IL-12p35 had exacerbated disease and symptoms in EAE (Gran, et al., 2002).

1.3.2.2 Th17 cells

Th17 cells are a novel subset of T helper cells discovered in 2003. They are distinct from Th1 and Th2 cells and the discovery of its role in causing and sustaining immune-mediated tissue injury has generated tremendous excitement in shifting immunologists’ view on tissue injury in organ-specific autoimmunity away from the Th1 paradigm which has been dominant for the past two decades (Steinman, 2007). Human Th17 cells differentiate from activated T cells in the presence of transforming growth factor (TGF)-beta, IL-1b, and IL-2 in combination with IL-6, IL-21, or IL-23 (Manel, et al., 2008). Interestingly, IL-23 is another member of the p40 cytokine family, which shares the same p40 subunit as IL-12. Th17 cells are characterized by the production of IL-17 (IL-17A), a proinflammatory cytokine which enhances T cell priming and stimulates the production of proinflammatory molecules. Th17 cells also produce other effector cytokines such as IL-17F, IL-21, and IL-22, which mediate host defensive mechanisms to
infections, as well as the pathogenesis of many autoimmune diseases (Ouyang, et al., 2008). IL-17 contributes to inflammation through the induction of neutrophil-recruiting chemokines (CXCL1, CXCL2, CXCL8).

Th17 cells are pathogenic in many animal models of autoimmune disease, including in EAE (Steinman, 2007). In one model, IL-12 was important for Th1 development but not crucial for the development of EAE (Cua, et al., 2003). In addition, IL-23 was identified as a critical cytokine for autoimmune inflammation in the brain (Zhang, et al., 2003).

There is also evidence of Th17 cell involvement in MS patients. Th17 cells are more abundant in RRMS patients during relapse in comparison with patients in remission and are more abundant in clinically isolated syndrome (CIS) patients with acute symptoms in comparison with patients with no acute symptoms. Th17 cell clones from CSF and peripheral blood of MS patients had higher levels of T cell activation markers (CD5, CD69, CD2, MHCII) and more abundant adhesion molecules (CD49d,CD6) compared to Th1 cell clones. Th17 cells may comprise a higher pathogenic potential as they are more proliferative and more difficult to suppress compared to Th1 cells (Brucklacher-Waldert, 2009). Th17 cells can be a relevant pathogenic subset in MS and provides an attractive therapeutic target (Juszczak and Głabiński, 2009).

Nonetheless, a phase II trial of ustekinumab, a humanized antibody against IL-12/23p40 failed to show clinical or radiological effects in RRMS compared to placebo controls (Segal, et al., 2008). The lack of efficacy could be partially due to the study’s inclusion of patients with advanced disease or the antibodies’ inability to enter the blood brain barrier to neutralize the interleukins at the site of inflammation (Longbrake and Racke, 2009).

1.3.2.3 Th2 cells
Th2 cells could be involved in the immunopathogenesis of MS since they are responsible for the activation of B cells, which have been generally accepted to play a role in MS.

In the presence of IL-4, naïve T cells differentiate into Th2 cells providing IL-4, IL-5, IL-6, IL-10, and IL-13 to B cells to launch the humoral immune response in fighting extracellular pathogens. It is generally accepted that Th1 and Th2 cytokines are mutually inhibitory at the T cell level and mechanisms of MS therapies have often been attributed to the shift of the Th1 response to the Th2 response (Gor, et al., 2003).
Even though it is well established that the Th2 cytokine IL-10 inhibits naïve T cells towards the Th1 phenotype, IL-4 and IL-13 have been demonstrated to enhance IL-12 production from macrophages and dendritic cells, thereby promoting the development of Th1 cells (Bullens, et al., 2001). Additionally, cotransfer of Th2 cells that secrete IL-4, IL-10 and low levels of TNF-alpha did not modify disease induced by adoptive transfer of MBP-specific Th1 cells that secrete IFN-gamma, TNF-alpha and low levels of IL-10 in one EAE model (Lafaille, et al., 1997), whereas T regulatory cells secreting IL-10 were able to suppress EAE in another model (Stohlman, et al., 1999), suggesting that it may not be a Th2 response but the ability to secrete IL-10 that is critical in suppression of EAE (Gor, et al., 2003).

### 1.3.2.4 B cells and pathogenic autoantibodies in MS

The beneficial effects of B cell depletion therapy (Hauser, et al., 2008) and plasmapheresis (Keegan, et al., 2005) has sparked a renewed interested of the potential role of B cells and autoantibodies in MS. Oligoclonal immunoglobulin G (IgG) bands (OCB) are present in the cerebral spinal fluid (CSF) of the majority of MS patients and the presence of OCBs has remained as a modern diagnostic procedure to obtain support for the diagnosis of MS (Link and Huang, 2006). CSF B cells produce the oligoclonal Ig bands in MS (Obermeier, et al., 2008) and the immunoglobulins are hypothesized to target myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated glycoprotein (MAG) (reviewed by Fraussen, et al., 2009) although this has been contradicted in other studies (Owens, et al., 2009). Oligoclonal CSF immunoglobulins from MS patients were also found to bind EBV proteins (Cepok, et al., 2005).

In neuromyelitis optica (NMO), a relapsing remitting demyelinating disease akin to MS, a serum antibody targeting aquaporin4 found on astrocytes leads to the loss of astrocytes and the multifocal demyelination seen in the disease (reviewed by Cayrol, et al., 2009; Prineas, 2009). Likewise, the demyelination observed in MS may not be starting point of the disease, as an acute loss of astrocytes or oligodendrocytes could also result in demyelination as seen in MS.

Various candidate antigens have been identified for MS, but the specificity of the autoreactive B cells and antibodies remain unclear and their relevance in disease process remains to be elucidated (reviewed by Fraussen, et al., 2009). In short, different myelin and non-myelin antigens may be targeted in different patients and contribute to the heterogeneity of the disease.
1.3.2.5 CD8+ T cells
CD8+ T cells recognize endogenous peptides presented by MHCI which is present in almost all nucleated cells. To protect against viral infections, they secrete cytokines IFN-gamma and TNF-alpha and cytotoxic mediators such as granzyme B and perforin to kill infected cells. Several lines of evidence show that CD8+ T cells have a role in MS. It is the most frequent subset of T cells found in chronic lesions (Hauser, et al., 1986; Booss, et al., 1983; Babbe, et al., 2000; Kivisäkk, et al., 2003). Effector CD8+ T cells can directly damage CNS and interestingly, axonal damage in lesions correlate well with CD8+ T cells infiltrates (Bitsch, et al., 2000; Kuhlmann, et al., 2002). Moreover, in MS therapies targeting T cells, the monoclonal antibodies that deplete all T cells had a better therapeutic benefit than the monoclonal antibodies targeting CD4+ T cells or against CD4+ T cell cytokines. No benefit was observed in MS patients receiving anti-CD4 MAb cM-T412 (van Oosten, et al., 1997), or a monoclonal antibody that neutralizes IL-12/IL-23 p40 (ustekinumab) (Segal, et al., 2008). By contrast, anti-CD52 monoclonal antibody (alemtuzumab) which targets both CD8+ and CD4+ T cells reduced relapses and new lesions (CAMMS223 Trial Investigators, et al., 2008). The trial for fingolimod (FTY-720), which targets the sphingosine 1-phosphate receptor-1 (S1P1) on both CD4+ and CD8+ T cells to prevent their egress from lymph nodes was also positive (Kappos, et al., 2006; Kappos, et al., 2010).
Natalizumab, which prevents both CD4+ and CD8+ T cells entry into the CNS by inhibiting very late antigen-4 and other integrins was successful as well (Polman, et al., 2006). Altogether, these indicate that CD8+ T cells play a role in MS pathogenesis (Friese and Fugger, 2009).

1.4 Treatment of MS
MS is a complex disease with heterogeneity that is incompletely understood and MS patients show a high degree of clinical variability. There is no single therapeutic target for MS. Treatment for affected patients requires drugs for alleviating symptoms, managing acute attacks, as well as disease modifying drugs (DMDs) for modifying the natural history of the disease. Several effective DMDs are currently available and others are in development for MS. These drugs differ in efficacy, safety profiles and mechanism of action.

1.4.1 Management of acute attacks

1.4.1.1 Corticosteroids
To manage acute attacks, high dose short-term oral prednisone or intravenous (IV) methylprednisolone for 3-5 days is used to reduce inflammation, shorten relapse duration and
hasten recovery (Milligan, et al., 1987; Tumani, 2009). Side effects of corticosteroids include possible changes in behavioural effects, sleep derangements, hypertension, diabetes, lipid derangements, gastritis and reflux, as well as edema (Tumani, 2009). Disease modifying effects of corticosteroids are not clear and more data is needed to determine its influence on new relapses and its effects on long term disability (Myhr and Mellgren, 2009, Tumani, 2009).

1.4.1.2 Plasma exchange

Plasma exchange removes serum and replaces it with a solution of albumin. This process removes large molecular weight particles from plasma. In the process, removing large molecules such as autoantibodies, immune complexes, cytokines and other inflammatory mediators is thought to be its principal mechanism of action. In one study studying plasma exchange in acute relapses, Weiner and colleagues compared 11 treatment cycles of plasma or sham exchange for 8 weeks. Clinical improvement was observed 2-4 weeks after plasma exchange, but the long term effects were unclear at one year (Weiner, et al., 1989). Weinshenker and colleagues also reported that plasma exchange was beneficial for patients with acute relapses. In their study, substantial improvement was seen in patients receiving seven treatment sessions within six weeks of symptom onset, but not in the sham treatment group (Weinshenker, et al., 1999). Plasma exchange was effective in improving visual activity in optic neuritis in 10 patients with MS or clinically isolated syndromes (Ruprecht, et al., 2004).

The effect of plasma exchange on chronic progressive MS is unclear (Tumani, 2008). In a randomized, controlled, double-blind trial involving 54 patients with chronic progressive MS, Khatri and colleagues reported that clinical improvement by expanded disability status scale (EDSS) was seen in the plasma exchange group after five months (Khatri, et al., 1985). However, in a study by the Canadian Cooperative Multiple Sclerosis Study Group, there were no significant differences in EDSS at 6 or 12 months between the three study groups: intravenous cyclophosphamide and oral prednisone; daily oral cyclophosphamide, alternate day prednisone (22 weeks) and weekly plasma exchange (20 weeks); or placebo medications and sham plasma exchange (The Canadian Cooperative Multiple Sclerosis Study Group, 1991). The Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology and the MS Council for Clinical Practice Guidelines concluded that plasma exchange "is of little or no value in the treatment of progressive MS" (Goodin, et al., 2002).

Side effects of plasma exchange include possible complications related to the use of central venous access, for example infection, septicaemia, thrombosis or pneumothorax. Alterations in
acid-base homeostasis or hypocalcaemia may result from citrate infused for anticoagulation or fresh-frozen plasma. Hypocalcaemia may lead to paresthesia, muscle cramps or cardiac arrhythmias (Lehmann, et al., 2006). These complications need to be carefully considered, especially when alternative treatments exist (Lehmann, et al., 2006).

1.4.2 Approved disease modifying therapies

1.4.2.1 Interferon-beta (IFN-beta)

Interferon-beta has been the first approved disease-modifying drug (DMD) and changed the natural history of patients with RRMS. In a small study, Jacobs and colleagues showed that intrathecal administered natural fibroblast IFN-beta reduced relapse rates in MS patients compared to controls (Jacobs, et al., 1982; Jacobs, et al., 1985). In spite of this, use of IFN-beta was limited because of the difficulty in obtaining natural interferons. As modern biotechnology and cell culture techniques advanced, biopharmaceuticals could be produced in large quantities and abundant recombinant IFN-beta became available for Phase III trials.

Three forms of recombinant IFN-beta are currently available and marketed as a first line treatment of multiple sclerosis. IFN-beta-1b, Betaseron® (Bayer, Canada) or Extavia® (Novartis, Canada) is given subcutaneously (SC) every other day at 8x10^6 International Units (IU) or (250μg). IFN-beta-1a is available as Avonex® (Biogen Idec, Canada) or Rebif® (EMDSerono, Canada). Avonex® is injected intramuscularly (IM) once a week at 6x10^6 IU (30μg) and Rebif® is administered SC thrice weekly at 6x10^6 IU or 12x10^6 IU (22μg or 44μg). IFN-beta-1b is a recombinant product from Escherichia coli (E. coli), whereas IFN-beta-1a is produced by a Chinese Hamster Ovary (CHO) cell line, a mammalian cell line. IFN-beta-1a is a glycosylated product and shares the same amino acid sequence as the IFN-beta made by natural human fibroblasts. In contrast, IFN-beta-1b is non-glycosylated and has a serine substituting for cysteine at position 17, as well as one amino acid deletion at methionine-1. This has implications in the immunogenicity of the drug as well as the solubility of the drug.

All three IFN-beta products have been shown to reduce relapse rates and disease burden by magnetic resonance imaging (MRI) in large multi-center, double-blinded, placebo controlled trials.

Betaseron® was the first drug shown to alter the natural history of MS patients. In the Betaseron® pivotal trial, patients were randomized 1:1:1 to receive 8 million international units (MIU), 1.6MIU, or placebo. Patients in the 8MIU arm had significantly reduced frequencies of
moderate and severe relapses as well as reduced accumulation of MRI abnormalities compared to placebo controls. A trend towards reduced disability was also observed, although the difference was not significant (The IFNβ Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group, 1993; Paty, et al., 1993).

Similarly, a randomized, double-blinded, placebo-controlled study was used to assess whether intramuscular IFN-beta-1a (Avonex®) can slow the progressive, irreversible neurological disability of relapsing MS. Compared to placebo controls, Avonex®-treated patients had reduced accumulation of permanent physical disability, exacerbation frequency, and disease activity measured by gadolinium-enhanced lesions on brain MRI (Jacobs, et al., 1996; Simon, et al., 1998).

A third randomized, double-blind, placebo-controlled study investigated the effects of subcutaneous interferon beta-1a (Rebif®). Patients were either given Rebif® 44μg, Rebif® 22μg, or placebo. Both treatment groups had lower relapse rates, delayed progression in disability, and lower accumulation of burden of disease and number of active lesions on MRI in a dose-related manner (PRISMS Study Group and the University of British Columbia MS/MRI Analysis Group, 2001).

1.4.2.1.1 The type I interferon signalling pathway

Interferons (IFNs) are a group of naturally occurring proteins produced by many mammalian cells. They influence normal and tumour cell growth, differentiation and death, modulate the immune response and regulate resistance to viral infections. There are four major groups of interferons: IFN-alpha, IFN-beta, IFN-gamma, and IFN-lambda. IFN-alpha and IFN-beta comprise the type I interferons. Both IFN-alpha and IFN-beta bioactivity are mediated through interactions with IFN-alpha receptor heterodimers (IFNAR1 and IFNAR2) on cell surfaces.

Following binding, signalling is initiated by pre-associated tyrosine kinases JAK1 and TYK2, which phosphorylate IFNAR1 and leads to the recruitment and phosphorylation of the signal transducers and activators of transcription (STATs). Signal is propagated when phosphorylated STATs form homodimers or heterodimers which are translocated into the nucleus. In the nucleus, these complexes bind to specific DNA sequences of the interferon stimulated response element (ISRE) promoter element to transcribe and express IFN-stimulated genes (ISGs), which underlie the antiviral, antitumor and immunoregulatory effects of IFN.
IFN-beta and IFN-alpha have overlapping as well as distinct biological activities. Although both interferons bind the same receptor, differences in receptor binding result in distinct signal transduction processes and hence the differences in the expression of ISGs (Stark, et al., 1998).

1.4.2.1.2 IFN-beta mechanism of action
The rationale for IFN-beta therapy nests in the concept that MS may be caused by a viral agent, and the antiviral activity of IFN-beta could benefit MS patients. The principal mechanisms by which IFN-beta drugs exert their effect is unclear and not completely understood and is attributed to the immunomodulatory rather than the antiviral or antiproliferative properties of the drug.

The immunomodulatory effects of IFN-beta include the inhibition of lymphocyte trafficking to the CNS, enhancement of suppressor T cell activity, reduction of pro-inflammatory cytokine production, upregulation of anti-inflammatory cytokines, as well as the down-regulation of antigen presentation and co-stimulatory molecules important for T cell activation. These effects are mediated by products of ISGs but only several ISGs have been assigned putative roles in mediating the therapeutic effects of the drug.

1.4.2.1.2.1 Effects on lymphocyte trafficking
The overall effect of IFN-beta on lymphocyte trafficking is a reduction of lymphocyte influx into the CNS to propagate inflammation.

To egress from lymph nodes to the vascular lumen, it is essential for sphingosine 1-phosphate (S1P) to interact with its receptor sphingosine 1-phosphate receptor (S1PR1) on lymphocytes. IFN-beta inhibits lymphocyte responsiveness to S1P by inducing CD69 expression on the T cell surface, which forms a complex with and negatively regulates S1P1 to suppress lymphocyte exit from lymph nodes (Shiow, et al., 2006).

To migrate from the vascular lumen into tissues, T cells express the adhesion molecule VLA-4, which interacts with VCAM-1 (vascular cell adhesion molecule-1) on the vascular endothelium to allow attachment to enter the endothelium. After anchoring, T-cells secrete metalloproteinases (MMPs) which degrade the extracellular matrix, allowing T-cells to enter the blood brain barrier. IFN-beta decreases the expression of VLA-4, VCAM-1 and improves the integrity of the blood brain barrier (Calabresi, et al., 1997). IFN-beta also increases plasma soluble vascular adhesion molecule (sVCAM), which acts as a decoy mechanism preventing
VLA-4 from binding to cell surface VCAM-1 (Calabresi, et al., 1997). Furthermore, IFN-beta reduces the expression of MMP9 in activated lymphocytes and upregulates TIMP-1, tissue inhibitor of metalloproteinase-1, which inhibits MMP9 from degrading the extra cellular matrix. In fact, the authors of one study concluded that the ratio of MMP-9/TIMP-1 may predict MRI activity in SPMS (Waubant, et al., 2003).

1.4.2.1.2.2 Effects on cytokine expression and T cell activation
Treatment with IFN-beta reduces the production of pro-inflammatory cytokines IFN-gamma, IL-12 and IL-23 and increases the production of anti-inflammatory cytokine IL-10 (Ramgolam, et al., 2009; Graber, et al., 2007). The reduction of pro-inflammatory cytokines reduces cell mediated immune response. The upregulation of anti-inflammatory cytokines promotes the conversion of naïve T cells to regulatory T cells to suppress the proliferation of self-reactive T-cells. The reduction of IFN-gamma suppresses MHC class II molecule expression on macrophages and monocytes and reduces antigen presentation to T cells (Chofflon, 2005). Expression of co-stimulatory molecules is also reduced by IFN-beta (Genç, et al., 1997; Zhang, et al., 2002). Altogether, IFN-beta decreases T cell activation (reviewed by Weinstock-Guttman, et al., 2008).

1.4.2.2 Glatiramer acetate (Copaxone®)
Glatiramer acetate (GA), also known as copolymer-1, is a random mixture of synthetic polypeptide made of four amino acids: L-glutamic acid, L-lysine, L-alanine and L-tyrosine. It structurally resembles myelin basic protein (MBP) and is believed to work by shifting the pro-inflammatory Th1 response to an anti-inflammatory Th2 response. Glatiramer-specific Th2 cells are produced in the periphery and when they enter the CNS, they are activated by myelin-antigens to secrete cytokines which suppress inflammation. GA may also work as a neuroprotective agent by stimulating neurotrophin secretion in the CNS (Blanchette and Neuhaus, 2008).

Currently GA is approved for RRMS as well as for treating early, isolated neurological episodes associated with MS. GA is administered subcutaneously once daily at 20mg and has efficacy in reducing relapse rates and improving disability (Johnson, et al., 1995; Johnson, et al., 1998; Comi, et al., 2001) although effects on long term disability are less clear.

Head to head comparison of GA 20mg SC once daily with IFN-beta-1b 250μg or 500μg SC every other day showed no difference in the clinical outcome of patients with RRMS (O’Connor, et al., 2009). The two drugs have different adverse effects: flu-like symptoms were more
common in IFN-beta-1b treated patients, whereas injection-site reactions were more common in patients treated with GA.

1.4.2.3 Mitoxantrone (Novantrone®)
Mitoxantrone is an antineoplastic agent which inhibits DNA and RNA synthesis and hence has broad cytotoxic effects on B cells, T cells and macrophages, efficiently suppressing the pathogenic immune response in MS (Jain, 2000; Fox, 2006). Mitoxantrone is approved for patients with worsening relapsing-remitting MS, progressive-relapsing MS or secondary-progressive MS and has been shown to reduce both attack and progression rates (Gonsette, 1996). In a randomized multi-centre study, Edan and colleagues studied the therapeutic effect of mitoxantrone (IV 20mg monthly) combined with methylprednisolone (IV 1g monthly) vs. methylprednisolone alone in MS patients with active disease. Blinded assessment of MRI data showed a significant lower number of enhancing lesions in the mitoxantrone treated group, and unblinded clinical assessment showed a significant improvement in EDSS score (Edan, et al., 1997). Given intravenously once every three months at 12mg/m², mitoxantrone offers an advantage for patients who find the self-injected therapies less acceptable. Nonetheless, potential cardiotoxicity limits the use of the agent for two to three years as the recommended maximum cumulative dose is 140 mg/m² (Scott and Figgitt, 2004).

1.4.2.4 Natalizumab (Tysabri®)
Natalizumab is a monoclonal humanized antibody which binds to alpha4 integrin, a glycoprotein present on the surface of inflammatory cells, and prevents their transmigration into areas of inflammation in the CNS (Horga and Horga de la Parte, 2007). Two large, multi-center, phase III trials has shown efficacy of natalizumab in reducing the rate of relapses as well as in reducing the risk of sustained progression of disability (Polman, et al., 2006; Rudick, et al., 2006). There is an increased risk of progressive multifocal leukoencephalopathy (PML) in natalizumab treated patients. PML is a demyelinating disease of the white matter of the human brain caused by lytic infection of oligodendrocytes with the human polyomavirus JC virus. PML is a rare disease which often occurs in severely immunosuppressed individuals and usually lead to death or severe disability (Major, 2009; Major, 2010). Two cases of PML were reported in the SENTINEL trial, which compared natalizumab alone vs. natalizumab and IFN-beta-1a in combination (Rudick, 2006; Goodin, et al., 2008). Both patients were in the IFN-beta-1a and natalizumab combination arm (Rudick, 2006). A third PML case was in the post mortem brain tissue of patient with Crohn’s disease who was treated with natalizumab (Van Assche, et al.,
In pooled clinical trial cohort, PML risk in natalizumab is estimated to be 1 in 1000 patients treated for 17.9 months (Goodin, et al., 2008). From July 2006 to Feb 10 2010, a total of 31 cases of PML in an estimated 66,000 natalizumab-treated patients have been reported to the Food and Drug Administration (FDA) (US FDA, Feb 2010). The overall worldwide cumulative rate of PML in patients who have received at least 1 infusion is 0.5 per 1,000 patients, and the worldwide cumulative rate of PML in patients who have received at least 24 infusions is 1.3 per 1,000 patients (US FDA, Feb 2010). Because of the potential increased risk of PML, it is recommended by the American Academy of Neurology (AAN) that natalizumab be reserved for use in selected patients with RRMS who have failed other therapies or in those who have a particularly aggressive initial disease course (Goodin, et al., 2008).

1.4.3 Oral therapies currently in development

The current approved immunomodulatory therapies for MS require parenteral administration. Clearly, an orally administered therapy with equal efficacy will have a better general acceptance as well as drug adherence amongst patients. Several oral therapies are in phase III trials for MS. Fingolimod and Laquinimod are novel immunosuppressive agents especially being developed for MS. Teriflunomide, cladribine, dimethyl fumarate and minocycline are being investigated due to their immunomodulatory effects in other diseases and have better established safety profiles.

1.4.3.1 Fingolimod (FTY720)

Fingolimod (FTY720) is a pro-drug which becomes phosphorylated in vivo. Phospho-fingolimod (P-FTY720), the active form, is a structural analogue of sphingosine and a sphingosine-1-phosphate receptor (S1PR1) agonist. Fingolimod causes lymphopenia by preventing egress of lymphocytes from lymph nodes hence prevents activated T cells from entering the CNS (Hiestand, et al., 2008).

The 6-month phase II trial of oral fingolimod compared 5.0mg, 1.25mg once daily or placebo in MS patients with RRMS or SMPS. Patients who received either dose showed a reduction in relapse rate and MRI-related measurement outcomes compared to placebo. More patients in the fingolimod groups remained relapse-free for the duration of the study compared to the placebo group (Kappos, et al., 2006). The higher dose offered no efficacy advantage and a less favourable safety profile compared to the lower dose. In subsequent phase III trials, a lower dose was tried.
Two phase III trials were conducted to show the efficacy of oral fingolimod. The two-year FREEDOMS trial compared oral fingolimod 0.5 mg or 1.25 mg once daily vs. placebo, and the one-year TRANSFORMS trial compared oral fingolimod 0.5 mg or 1.25 mg once daily compared to IFN-beta-1a 30ug IM once weekly. In the FREEDOMS trial comparing fingolimod and placebo, fingolimod reduced relapse rates, reduced the risk of disability progression, as well as improved MRI-related measurement outcomes and clinical disease activity in patients with RRMS (Kappos, et al., 2010). In the TRANSFORMS trial comparing fingolimod and IFN-beta, fingolimod was more effective in reducing relapses than IFN-beta-1a (Cohen, et al., 2010). Both studies showed no differences in efficacy between the two doses of fingolimod, whereas adverse events appeared to be less frequent with the 0.5 mg dose than with the 1.25 mg dose. The dose which optimizes drug benefit while minimizing adverse events still needs to be explored.

Adverse events among patients receiving fingolimod included an initial reduction in heart rate and atrioventricular conduction block at the time of fingolimod initiation, which may require close patient monitoring at the initiation of treatment. Macular edema, elevated liver-enzyme levels, mild hypertension and lower respiratory tract infections were also more common in patients receiving fingolimod (Kappos, et al., 2010). Herpesvirus infection may also be a concern in fingolimod treatment. In the FREEDOMS study, overall incidence of herpesvirus infection were similar in the three study groups; however, two cases that were classified as serious adverse events occurred in patients receiving fingolimod: one case of genital herpes in a patient from the 1.25mg fingolimod group, one case of herpes simplex labialis in one patient from the 0.5mg fingolimod group. In the TRANSFORMS trial, two fatal cases of disseminated primary varicella zoster and herpes simplex encephalitis occurred group receiving the 1.25mg-dose of fingolimod (Cohen, et al., 2010). Reactivation of latent herpes is a potential risk with immunomodulatory therapy. Long-term follow-up on the cardiovascular effects, the risk of infection and cancer are essential to assess the benefits and risks of this new treatment.

**1.4.3.2 Cladribine**

Cladribine is a lymphocyte depleting therapy. It is an adenosine analogue which incorporates into the DNA of rapidly proliferating B and T lymphocyte subpopulations. Lymphocytes are unable to effectively metabolize cladribine and accumulation of its metabolite 2-chlorodeoxyadenosine triphosphate results in selective lymphocyte depletion as cellular metabolism and DNA synthesis and repair is disrupted (Brousil, et al., 2006; Giovannoni, et al., 2010). Parenteral cladribine has a well-characterized safety profile from more than 15 years of
its use in hairy-cell leukemia and other haematological malignancies (Hartung, et al., 2010). In MS, cladribine is able to cross the BBB and can act on cells both in the periphery and the CNS. An annual, short dosing regimen is possible for cladribine because the effects of lymphocyte depletion can be sustained. This offers an additional appeal to patients (Hartung, et al., 2010).

The Cladribine Tablets Treating Multiple Sclerosis Orally (CLARITY) study is a phase III trial which assessed the efficacy of oral cladribine tablets in patients with RRMS. The 96-week study involved RRMS patients who were assigned to receive one of two cumulative doses (3.5mg or 5.25 mg per kilogram of body weight) of cladribine tablets or matching placebo, which was given as short courses consisting of one or two 10mg cladribine tablets or placebo given once daily during the first 4 or 5 days of a 28-day period. In the first 48 week period, the four courses were given on day 1, weeks 5, 9 and 13. The 5.25mg group received four courses of cladribine, the 3.5mg group received two courses of cladribine followed by two courses of placebo, and the placebo group received four courses of placebo. In the second 48 week period, both cladribine groups received two courses of cladribine and the placebo received two courses of placebo, which started at weeks 48 and 52. Compared to the placebo group, there was a significantly lower annualized relapse rate, a higher relapse-free rate, and a lower risk of 3-month sustained progression of disability, and reductions in the brain lesion count on MRI in both cladribine groups. Lymphocytopenia, herpes zoster and neoplasms were more frequent in the cladribine groups. This study showed that short courses of oral cladribine for only 8 to 20 days per year was beneficial for patients with RRMS (Giovannoni, et al., 2010), however, the optimal dosage and regimen still needs to be explored to minimize adverse events while retaining the beneficial effects of the drug. A phase III trial is underway to evaluate the effect of 2 other dosage regimens in CIS patients (ORACLE MS) and a phase II trial is ongoing to study cladribine as an add-on therapy to IFN-beta (ONWARD) (US National Institutes of Health, April 2010).

1.4.3.3 Teriflunomide

Teriflunomide is the active metabolite of leflunomide, an immunosuppressant which has been approved for the treatment of rheumatoid arthritis since 1998 (Tallantyre, 2008; Warnke, et al., 2009). It acts by inhibiting an enzyme important for de novo pyrimidine nucleotide synthesis in active lymphocytes. There is evidence that inhibiting de novo pyrimidine synthesis inhibits Th1 activation and favours the Th2 immune response (Dimitrova, et al., 2002). Besides inhibiting DNA/RNA synthesis, teriflunomide may also impair phospholipid synthesis and protein glycosylation, impairing function of lipid messengers and cell surface molecules (Herrmann, et al., 2000).
A phase II trial has been completed for oral teriflunomide and was published in 2006 (O’Connor, et al., 2006). RRMS and SPMS patients with relapses were randomized to receive placebo, 7mg or 14mg teriflunomide oral once daily. Teriflunomide was safe and well tolerated. Teriflunomide effectively reduced MRI lesions in relapsing MS and significantly lowered disability increase in the 14mg/day treatment group. There was also a non-significant trend towards lower annualized relapse rates.

Two phase III trial comparing placebo, 7mg or 14mg teriflunomide oral once daily in patients with relapsing MS (TOWER) or with CIS (TOPIC) are underway and due for completion in September 2011 and October 2012. Two other phase III trials are underway. One studies how teriflunomide affect the frequency of relapses and the accumulation of disability in patients with MS (TEMSO) and one compares IFN-beta-1a (Rebif®) vs. teriflunomide (US National Institutes of Health, 2009). Lastly, phase II trials are currently in progress to compare combination therapy of teriflunomide with GA or with IFN-beta.

1.4.3.4 Laquinimod

Laquinimod is another oral therapy currently under development. It is a synthetic immunomodulator and a derivative of roquinimex (linomide), which showed positive results in reducing clinical and MRI activity in phase II/III trials, but was discontinued due to serious cardiovascular events (Weiner, 2008). Laquinimod is thought to work by shifting the proinflammatory Th1 response to the anti-inflammatory Th2 response. In a phase IIb trial, 0.3 and 0.6 mg oral laquinimod daily reduced new MRI lesions in patients with RRMS (Comi, et al., 2008). Currently, two phase III trials are in progress for Laquinimod and it may become an alternative for the injectable first line therapies for relapsing MS (Preiningerova, 2009).

1.4.3.5 Dimethyl fumarate and BG12

Dimethyl fumarate (DMF) is a well established agent for the treatment of psoriasis and has been in use since 1959 (Schweckendiek, 1959; Tallantyre, et al., 2008). Although the mechanism of action of dimethyl fumarate is unclear, there is evidence that it can reduce CD4+ and CD8+ lymphocytes by inducing apoptosis and shift the cytokine profile from a Th1 to a Th2 profile. Another hypothesis is that DMF interferes with cellular redox systems to inhibit NF-κB translocation into the nucleus thereby reducing NF-κB dependent expression of inflammatory chemokines, cytokines and adhesion molecules (Moharregh-Khiabani, et al., 2009).

BG12 is a modified fumaric acid ester, an oral fumarate formulation being investigated for RRMS. In a phase II study, oral fumarate was shown to significantly reduce the number of
gadolinium enhancing lesions after 24 weeks of treatment compared to placebo. There was also a trend towards reduced annualised relapse rate for the fumarate-treated group (Kappos, et al., 2008). Further phase III studies have been started to explore the long-term efficacy of fumaric acid esters (Moharregh-Khiabani, et al., 2009).

1.4.3.6 Minocycline
Minocycline is a broad spectrum tetracycline antibiotic which is being under investigation for its neuroprotective and anti-inflammatory effects (Maier, et al., 2007; Giuliani, et al., 2005, Orsucci, et al., 2009). Minocycline is generally administered twice daily at 100mg and is currently used to treat acne and other skin infections as well as Lyme disease. It is also used as a treatment for mild rheumatoid arthritis.

In a recently completed phase II study comparing GA/placebo vs. GA/minocycline. RRMS patients receiving GA/minocycline tended to have better MRI outcomes and a lower risk of new relapses although this did not reach significance. The authors suggested that combination therapy could be beneficial for patients (Metz, et al., 2009). A phase II trial is investigating minocycline as add-on to IFN-beta-1a (Rebif®) in RRMS and a phase III trial is underway to investigate the effect of minocycline in reducing the conversion of CIS to McDonald Criteria MS (US National Institute of Health, 2010).

1.4.4 Monoclonal antibody therapies in development

1.4.4.1 Alemtuzumab (Campath-1H)
Alemtuzumab (Campath-1H) is a humanized monoclonal antibody that is directed against CD52. CD52 is a cell surface glycoprotein with an unknown function that is found on lymphocytes and monocytes but not on haematological precursors. Administration of the antibody results in a rapid, acute, but long-lasting lymphopenia, which is the rationale for using alemtuzumab as an MS treatment. Surprisingly, continued use of alemtuzumab does not greatly increase the risk of infections attributable to immunosuppression (Jones and Coles, 2009). The mechanism of action of alemtuzumab could hence be due to the homeostatic responses it induces in the immune cell repertoire, reducing memory T-cells and restoring T regulatory cells (CD4+ CD25hi FoxP3+ cells), providing a tolerogenic environment for developing lymphocytes (Jones and Coles, 2009).

Alemtuzumab was more effective than SC interferon beta-1a 44μg TIW in reducing the rate of sustained accumulation of disability, lesion burden and annualized rate of relapse in a phase II
trial (CAMMS223 Trial Investigators, et al., 2008). However, it was associated with considerable adverse effects resulting from high anti-inflammatory potential of the drug; 10-30% patients developed a novel autoimmune disease after treatment. In the CAMMS223 trial, three patients developed immune thrombocytopenic purpura (ITP) during the study, one of which whom died, and one case of Goodpasture syndrome was reported. There was also a higher frequency of thyroid-directed antibodies and thyroid associated disease in the treatment group vs. the placebo group (reviewed by Wiendl and Hohlfeld, 2009).

1.4.4.2 Rituximab and anti-CD20 antibody therapy

Rituximab is a chimeric monoclonal antibody against CD20, which is found on B cells. Infusion of the chimeric antibody results in B-cell depletion and rituximab was originally approved for non-Hodgkin’s lymphoma in 1999. The antibody has also been used to treat rheumatoid arthritis and has been used off-label for other autoimmune diseases with B cell involvement such as systemic lupus erythematosus and autoimmune anaemia. In the phase II trial of rituximab for RRMS, rituximab rapidly reduced acute disease activity as assessed by MRI. However, results of a phase III trial for PPMS were negative.

Second generation anti-CD20 monoclonal antibodies, ocrelizumab and ofatumumab are currently in phase II trials for the treatment of multiple sclerosis (Hutas, 2008; Kausar, et al., 2009; Castillo, et al., 2009). These are fully human antibodies and thus have a better safety profile than rituximab.

1.4.4.3 Daclizumab

Daclizumab is a humanized monoclonal antibody that blocks CD25, the alpha subunit of the high affinity IL-2 receptor expressed on activated lymphocytes. Daclizumab inhibits clonal expansion of activated T-cells by inhibiting IL-2 from binding to its receptor but allows signalling via the intermediate affinity receptors. Daclizumab reduce both CD4+ and CD8+ T cells while expanding CD56 bright natural killer cells that produce IL-10.

Daclizumab is approved by the food and drug administration for the prevention of acute transplant rejection. Several trials have investigated daclizumab for MS as an add-on therapy to IFN-beta and have been shown to have a positive influence in MRI measurements and on relapse rates (Schippling and Martin, 2008). Recent results of a 24-week phase II randomized, double-blind, placebo-controlled trial with IFN-beta showed that daclizumab reduced the number of new or enlarged gadolinium contrast-enhancing lesions compared with IFN-beta alone (Wynn et al., 2010). An open-label baseline vs. treatment phase II clinical trial of daclizumab in
patients having MS with inadequate response to IFN-beta showed that daclizumab is effective as a monotherapy (Bielekova, et al., 2009). The authors suggested that a higher dose of daclizumab may be needed to achieve optimal therapeutic response. Daclizumab has not yet been approved for the treatment of MS and long-term safety and efficacy remain unclear. As we anticipate results for phase II trials, more trials are warranted to study the use of daclizumab in multiple sclerosis.

1.5 Immunogenicity to therapeutic proteins

1.5.1 Therapeutic proteins

The use of therapeutic proteins has increased in the frequency and the number of use since the introduction of the first recombinant protein therapeutic - human insulin - in 1982. Currently, more than 130 different proteins or peptides have been approved by the FDA for clinical use and many more are currently in development. Proteins are involved in virtually all biological processes inside and outside of a cell. They catalyze biochemical reactions, form receptors and channels in membranes, provide intracellular and extracellular scaffolding and structural support, transport molecules within a cell or from one organ to another and serve as signalling molecules. Any defect in the estimated 25,000–40,000 different genes in the human genome may lead to defective proteins and disease, which could potentially be alleviated by a therapeutic protein. Proteins offer an advantage over small molecule drugs because they are highly specific and serve functions that cannot be replaced by small molecules. From a financial perspective, the complexity and unique form and functions of proteins often allow companies to obtain far-reaching patents for the protein therapeutic (reviewed by Leader, et al., 2008). Whilst the number of new molecular entities approved by the US FDA’s Center for Drug Evaluation and Research per year has decreased for the past 15 years, the number of new biologic license applications approved per year has remained similar. The increase in the percentage of total approvals for new biologics reflects the investment being made by the industry into biopharmaceuticals (Hughes, 2010).

1.5.1.1 Protein replacement

Therapeutic proteins are used in a wide range of conditions (reviewed by Leader, et al., 2008). A classic paradigm of protein therapeutics is in replacement therapy, where a specific endogenous protein is deficient and replaced by an exogenous protein to alleviate disease. Some examples are insulin in the treatment of diabetes, clothing factor VIII and factor IX in haemophiliacs and pancreatic enzymes in cystic fibrosis. Protein replacement therapy is also
common in metabolic enzyme deficiencies such as beta-glucocerebrosidase deficiency in Gaucher’s disease and alpha-galactosidase A deficiency in Fabry disease.

1.5.1.2 Protein augmentation
Protein therapeutics can also augment an existing pathway and has applications in haematopoiesis, fertility, immunoregulation, haemostasis and thrombosis, endocrine disorders and growth regulation. Some examples are erythropoietin (EPO) in anaemia, granulocyte-macrophage colony stimulating factor (GMC-SF) in leukaemia, myeloid reconstitution post-bone-marrow transplantation and HIV/AIDS (human immunodeficiency virus / acquired immune deficiency syndrome), human follicle-stimulating hormone (FSH) and human chorionic gonadotropin (HCG) in assisted reproduction, IFN-alpha in hepatitis B, hepatitis C, hairy cell leukaemia, Kaposi’s sarcoma, IFN-beta in MS, IFN-gamma in chronic granulomatous disease and severe osteoporosis, IL-2 in metastatic renal cell cancer and melanoma, tissue plasminogen activator (tPA) in myocardial infarction and stroke, and gonadotropin releasing hormone (GnRH) in precocious puberty.

1.5.1.3 Proteins that provide a novel function or activity
Protein therapeutics may also provide a novel function or activity. For example, botulinum toxin A and botulinum toxin B cleaves SNARE (Soluble N-ethylmaleimide Sensitive Fusion Attachment Protein Receptor) proteins at neuromuscular junctions to disrupt the SNARE complex to prevent acetylcholine release and causes flaccid paralysis. The toxin is used in many types of dystonia, and has cosmetic uses as well. Papain derived from Carica papaya fruit is used for debridement of necrotic tissue or liquefication of slough in acute and chronic lesions.

1.5.1.4 Proteins with a special targeting activity
Other protein therapeutics have a special targeting activity to interfere with a molecule or an organism involved in disease pathology or to deliver other compounds or proteins. These are often fusion proteins or monoclonal antibodies and are used in cancer, immunoregulation, transplantation, pulmonary disorders, infectious disease, haemostasis and thrombosis, as well as endocrine disorders. Well known examples are bevacizumab (binds to VEGFA, Vascular Endothelial Growth Factor A) in colorectal cancer and non-small-cell lung cancer, trastuzumab (Herceptin, binds to HER2/Neu, Human Epidermal Growth Factor Receptor 2) in breast cancer, infliximab (neutralizes TNF-alpha) in rheumatoid arthritis and Crohn’s disease, natalizumab (Tysabri, binds to alpha-4 subunit of alpha-4-beta-1 and alpha-4-beta-7 integrins) in multiple sclerosis, daclizumab (binds to alpha chain of CD25 of IL-2 receptor) in prophylaxis against
allograft rejection in patients receiving renal transplants, and enfuvirtide (Fuzeon), a peptide which inhibits HIV entry into host cells. Some biologics are coupled to radioactive, chemotherapeutic or toxic agents to deliver cytotoxic effects. For example, denileukin diftitox (Ontak) directs diphtheria toxin to cells expressing the IL-2 receptor to treat T-cell lymphomas with malignant cells which express the CD25 component of the IL-2 receptor.

1.5.1.5 Proteins in diagnostics
Additionally, therapeutic proteins are used in diagnostics. For example, in diagnostic imaging, indium-111-labelled anti-PSA (Prostate Specific Antigen) antibody is used to recognize intracellular PSA in prostate cancer, and technetium-labelled anti-CEA (Carcinogenic Embryonic Antigen) antibody is used for colon and breast cancer detection.

1.5.1.6 Protein vaccines
Lastly, protein therapeutics also includes protein vaccines such as the Hepatitis B vaccine which contains the Hepatitis B surface antigen (HBsAg) and the HPV (human papillomavirus virus) vaccine which contains major capsid proteins from four HPV strains. Vaccines are formulated to be highly immunogenic; injecting the protein fragment of the infectious agent induces neutralizing antibodies and memory cells against the protein fragment, which prepares the body to respond to the infectious agent by neutralizing it before it can enter cells and by recognizing and destroying infected cells.

1.6 Antibodies to therapeutic proteins
Whilst immunogenicity and antibodies are desired in protein vaccines, immunogenicity against other therapeutic proteins is not desirable. Neutralizing antibodies may hinder or enhance drug clearance, inactivate the drug to reduce drug efficiency or block the biological function of the endogenous protein. More safety issues arise if the protein induces a severe hypersensitivity or anaphylactic response.

Development of antibodies to therapeutic proteins was first described in diabetic individuals treated with recombinant, porcine and bovine insulin (Fireman, et al., 1982). High levels of anti-insulin IgG antibodies led to immune-mediated insulin resistance (Scherthaner, 1993). Other documented examples of antibody responses against therapeutic proteins include anti-erythropoietin antibodies which may lead to red cell aplasia (Casadevall, et al., 2002) and anti-factor VIII antibodies which may lead to acquired haemophilia (Ehrenforth, et al., 1992, Ananyeva, et al., 2009). Anti-IFN-alpha antibodies (McKenna and Oberg, 1997), anti-botulinum
toxin antibodies (Goschel et al., 1997), and anti-GM-CSF antibodies (Gribben, et al., 1990) interfere with drug efficiency.

Antibodies to biopharmaceuticals can be detrimental and need to be monitored during therapy. Multiple factors could contribute to the immunogenicity of therapeutic proteins, including drug formulation, route of administration and dose frequency. Patient genetic background and concomitant medication could also contribute to the individual’s immune response to protein therapy.

1.6.1 Neutralizing antibodies to interferon-beta
Neutralizing antibodies (NAbs) to IFN-beta were first described in a patient with a nasopharyngeal carcinoma treated with a preparation of human IFN-beta (Vallbracht, et al., 1981). NAbs to IFN-beta in treated MS patients were described in the pivotal trial of IFN-beta-1b (The IFNβ Multiple Sclerosis Study Group, 1993), and in the phase III trials for IM and SC IFN-beta-1a (Jacobs, et al., 1996; PRISMS Study Group, 1998). However, NAbs were not considered clinically relevant in these reports. In these studies, NAbs appeared in the first two year of therapy but the effect of NAbs do not appear until the third or fourth year of therapy. When the final outcome of the trials were published, NAbs were found to attenuate treatment effect on relapse and MRI measures (The IFN-beta Multiple Sclerosis Study Group and the UBC MS/MRI Analysis Group, 1995; Rudick, et al., 1998; Francis, et al., 2005).

1.6.1.1 Incidence of anti-IFN-beta antibodies
Varying anti-IFN-beta antibodies incidences have been reported and antibody positivity may be defined differently in each study. Some studies define NAb positivity as “once positive, always positive”, some studies define NAb positivity as having two or more consecutive positive NAb titres within the study period, while other studies may report the number of subjects with positive NAb at the end of the study period. Different NAb testing methods with different assay sensitivities may be used from one study to another. In clinical trial settings, the three companies manufacturing IFN-beta had antibody testing performed independently, hence direct comparison of the three IFN-beta products is not possible. Apart from clinical trial settings, not all IFN-beta patients are routinely tested for NAbs in practice, hence reports from independent laboratories may not represent the entire population.

1.6.1.1.1 NAb incidences in clinical trials
In the IFN-beta-1b pivotal trial, two year data reported that 11% of placebo sera, 47% of sera from the 1.6 MIU arm, and 45% patients from the 8 MIU arm were NAb positive (The IFNB
Multiple Sclerosis Study Group, 1993). The final outcome of the trial reported that 38% of patients in the 8 MIU arm were NAb positive (The IFNB Multiple Sclerosis Study Group and the UBC MS/MRI Analysis group, 1995). In this study, the median time to NAb positivity was 343 days and most patients became positive in year one.

In the PRISMS (Prevention of Relapses and Disability by Interferon-beta-1a Subcutaneously in Multiple Sclerosis) study, 23.8% and 12.5% patients were positive in the 22μg and 44μg group after 24 months of treatment (PRISMS Study Group, 1998). Using the “once positive, always positive” criteria, 23.7% and 14.3% patients were positive in the 22μg and the 44μg group after four years of treatment (The PRISMS Study Group and the UBC MS/MRI Analysis Group, 2001). The majority of patients who developed NAbs became positive within the first 12 months (60% and 80% for 22μg and 44μg). Eighty-five and 91% of NAb positive patients were positive at 18 months. Twenty percent patients who developed NAbs reverted to NAb negative status at the end of the study.

In the study for intramuscular IFN-beta-1a for disease progression in relapsing MS, 14% patients were positive at week 52, 21% were positive at week 78, and 22% were positive at week 104 (Jacobs, et al., 1996). In a subsequent open label safety extension study, NAb incidence over 24 months was approximately 5%, which was lower than in the phase III trial (Rudick, et al., 1998). This could partially be explained by the different batch number of IFN-beta-1a used in the two trials. Changes in manufacturing, purification and formulation may impact immunogenicity (Goelz and Walt, 2007). This will be an important issue as biosimilar products are being introduced into the market. Furthermore, the assays used to measure NAbs in the two studies were different: in the phase III trial, an anti-viral neutralization assay was used, where as in the phase IV trial, a new Myxovirus protein A Assay (MxA Assay) was used. For comparison, sera from week 104 in the phase III trial were retested using the original anti-viral neutralization assay as well as the new MxA assay. Seventeen percent were positive using the new assay, and 19% were positive using the original assay, supporting that the difference in NAb incidences not due to the different assays used (Rudick, et al., 1998). In the European IFN-beta-1a IM Dose-Comparison Study, only 1.8% of the patients in the 30μg group and 4.8% of the patients in the 60μg group were positive for NAbs (Kappos, et al., 2005).

**1.6.1.1.2 NAb incidences outside clinical trials**

Outside of clinical trials, NAb frequencies have been reported in several cohorts followed at MS clinics.
In an Italian cohort of 125 patients who were followed for up to 18 months, the prevalence of persistent NAb at month 18 was 31.6% for Betaseron® 250μg every other day, 18.7% for Rebif® 22μg SC thrice weekly and IM once or twice weekly, and 4% for 30μg Avonex® IM once weekly (Bertolotto, et al., 2002).

In a Danish cohort of 541 patients who were followed up to 60 months, NAb incidence at month 24 was 44% for Betaseron® 250μg every other day, 22% for Rebif® 22μg once weekly, 42% for Rebif® 22μg thrice weekly, and 8% for Avonex® 30μg once weekly (Sorensen, et al., 2003).

In a Canadian cohort of 262 patients treated with a single IFN-beta preparation for more than 3 years at the UBC MS Clinic, Boz and colleagues reported that 15.1% (18/119) patients treated with IFN-beta-1b were NAb positive, 12.2% (16/131) patients treated with SC IFN-beta-1a were NAb positive, but none of the 12 IM IFN-beta-1a treated patients had detectable NAb (Boz, et al., 2007).

### 1.6.1.2 Factors affecting immunogenicity of interferon-beta

IFN-beta-1b is considered more immunogenic than the IFN-beta-1a products and this has been shown in several studies (Perini, et al., 2001; Bertolotto, et al., 2002; Sorensen, et al., 2003; Boz, et al., 2007). The host cells in which the IFN-beta is produced could affect immunogenicity if host cell components are present in the final product. This is notable because IFN-beta-1b is made in bacterial cells, whereas IFN-beta-1a is made in mammalian cells. Furthermore, bacterial cells do not glycosylate proteins the same way as mammalian cells do. Glycosylation of IFN-beta is an important factor in the solubility of the protein (Karpusas, et al., 1998). Recombinant IFN-beta-1b produced in *E. coli* is a non-glycosylated product which could form protein aggregates in solution.

### 1.6.1.2.1 Route of administration, dose and administration frequency

It is generally accepted that subcutaneous preparations are more immunogenic than intramuscular preparations and this is evident in the Danish cohorts where NAb incidence was higher in the 22μg IFN-beta-1a (Rebif®) SC once weekly group compared to the 30μg IFN-beta-1a (Avonex®) IM once weekly group (Ross, et al., 2000; Sorensen, et al., 2003). This was also observed by Perini and colleagues who found that the rate of binding antibody development and the antibody titres were lower in the IFN-beta-1b 250μg IM once a week group versus the IFN-beta-1b 250μg SC every other day group (Perini, et al., 2001). On the other hand, Bertolotto and colleagues found that the incidence of NAb persistence were similar with the IM IFN-beta-1a (Rebif®) 22μg once or twice weekly and SC IFN-beta-1a (Rebif®) 22μg thrice weekly groups...
Farrell and Giovannoni commented that the evidence for the route of administration’s impact on immunogenicity is unclear because different products, different dose and dose frequencies have been used to compare the IM and SC routes in these studies (Farrell and Giovannoni, 2007). Frequent drug administration increased the rate of positive NAb samples in the Danish cohort which included patients on SC IFN-beta-1a 22μg thrice weekly and SC IFN-beta-1a 22μg once a week (Sorensen, et al., 2003).

Dose effects on immunogenicity are less clear. In the European IFN-beta-1a IM Dose-Comparison Study comparing 30μg and 60μg once weekly, NAb incidence was higher in the high dose group than in the low dose group (Kappos, et al., 2005). On the other hand, in the Once Weekly Interferon for MS (OWIMS) Study and the PRISMS Study where 22μg and 44μg were compared, NAb incidence was higher in the 22μg groups compared to the 44μg groups (PRISMS Study Group, 1999; The Once Weekly Interferon for MS Study Group, 1999).

1.6.1.2.2 Patient genetics

Barbosa and colleagues reported that there is an association between anti-IFN-beta antibody response and a common MHC class II allele (DRB1*0701) (Barbosa, et al., 2006). Another study found that HLA-DRB1*0401 and HLA-DRB1*0408 are associated with development of antibodies to IFN-beta during treatment (Hoffmann, et al., 2008). These HLA alleles may have a peptide-binding motif which promote binding and presentation of the immunogenic peptide and facilitate antibody development.

1.6.1.2.3 Concomitant medication

Pozzilli et al., showed that combination therapy of IFN-beta and methylprednisolone decreased the development of antibodies to IFN-beta-1b. In the study, 26.8% of patients in the IFN-beta-1b alone group developed NAbs during the study and only 12.1% patients receiving methylprednisolone in addition to IFN-beta-1b developed NAbs (Pozzilli, et al., 2002).

On the other hand, immunosuppression has no or little effect on IFN-beta bioactivity in NAb positive patients once NAbs has developed. Hesse and colleagues reported that monthly pulsed methylprednisolone therapy for six months did not affect NAb positive status or restore IFN-beta biological activity (Hesse, et al., 2009). Treatment with azathioprine and cyclic methylprednisone for six months also had no or little effect on IFN-beta bioactivity in NAb positive patients (Ravnborg, et al., 2009).
1.6.1.3 Clinical relevance of anti-interferon-beta antibodies

Given that the effects of lost drug efficiency is not seen until after the third or fourth year of treatment, most clinical trials for IFN-beta which are 2-3 year in length concluded that anti-interferon antibodies are not clinically relevant. In addition to study duration, another challenge to studying the effect of NAbs on IFN-beta treatment is in the lack of available MRI data outside of clinical trials.

Anti-IFN-beta antibodies were reported to reduce drug efficacy as evidenced by MRI and or clinical parameters for disease activity in the phase III trials for IFN-beta1b, and both IM and SC IFN-beta-1a (IFNB Multiple Sclerosis Study Group and the UBC MS/MRI Analysis Group, 1995; Rudick, et al., 1998; Francis, et al., 2005).

In the final outcome of the IFN-beta-1b pivotal trial, NAbs significantly attenuated treatment effect on exacerbation rate. At 18 months, patients who had more than 2 consecutive NAb positive tests had exacerbation rates similar to the placebo group. The number of enlarging MRI lesions significantly increased compared to those who were NAb negative (The IFNB Multiple Sclerosis Study Group and the UBC MS/MRI Analysis Group, 1996).

In the PRISMS trial, relapse and disability measures over four years were similar for patients who were NAb positive anytime during the study, but there were more relapses in the NAb positive group during years 3-4. Patients who were at anytime NAb positive had more median active lesions per MRI scan and more cumulative percent change in T2 lesion burden from baseline to years 2 and 4. Using an interval positive analysis method, where the NAb status at the end of each six month interval is considered, a significant difference was observed in the relapses rates between NAb positive and NAb negative group over four years as well as during years 3-4. More NAb negative patients were relapse free during years 3-4 (Francis, et al., 2005).

In the European Interferon beta-1a IM dose comparison study, patients who had two or more consecutive NAb tests showed higher relapse rates from months 12 to 48, higher rate of mean change (worsening) in EDSS score from baseline to month 48, greater number of T1 gadolinium-enhanced lesions at months 24 and 36, and greater accrual of new or enlarging T2 lesions from month 12 to months 24 and 36. This study showed that NAbs to IFN-beta-1a reduced the therapeutic benefits measured by relapses, MRI activity, as well as changes in EDSS score (Kappos, et al., 2005).
In the Danish cohort of IFN-beta treated patients followed for up to 60 months, disease activity increased in NAb positive patients compared to NAb negative patients. Relapse rates were significantly higher during NAb positive periods compared to NAb negative periods. Time to first relapse was significantly increased in patients who were NAb negative at 12 months. Presence of NAbs did not affect disease progression measured with EDSS. The authors concluded that the presence of NAbs reduces the clinical effect of the drug and change of treatment should be considered for NAb positive patients (Sorensen, et al., 2003).

In the Canadian post-marketing study, Boz and colleagues showed that anti-IFN-beta antibodies has an effect on MS relapse rates during years 3 and 4 of therapy and this was different for IFN-beta-1a and IFN-beta-1b. For IFN-beta-1b treated patients, relapse rates tended to be higher in the NAb positive group in year 3 and this almost reached significance, but the relapse did not differ in year 4 and later. For IFN-beta-1a treated patients, NAb positive patients tended to have more relapses in year 3, but in year 4, they had significantly more relapses than NAb negative patients (Boz, et al., 2007).

Anti-IFN-beta antibodies are associated with the loss of therapeutic efficiency for the most part; but it is also important to recognize that antibodies bind to both exogenous and endogenous IFN-beta and has potential for other side effects. Endogenous IFN-beta is important for its anti-tumour effects. Malignant melanoma in an MS patient with persistent NAbs to IFN-beta was described in a case report (Gibbs, et al., 2008).

1.6.2 Measuring anti-interferon-beta binding antibodies and neutralizing antibodies

Antibodies to IFN-beta are described as binding antibodies, or neutralizing antibodies dependent on the assay used to identify the antibody. Binding antibodies (BAbs) are recognized by binding assays such as enzyme linked immunosorbent assays (ELISA) and radioimmunoprecipitation assays (RIPA). These antibodies bind to IFN-beta, but do not necessarily block epitopes important for biological activity. Neutralizing antibodies bind to IFN-beta and block a biological response of interferon-beta. Some also designate antibodies as “non-neutralizing antibodies”, which describe binding antibodies which are not neutralizing antibodies.

1.6.2.1 Measuring anti-interferon-beta binding antibodies

Since all NAbs are BAbs, BAb assays are used as an economical method to screen to identify samples that need to be further tested for NAbs (Sorensen, et al., 2006). Additionally, the
appearance of BAbs precedes NAbs and can be used to monitor the development and affinity maturation of antibodies to IFN-beta.

1.6.2.1.1 **Enzyme-linked immunosorbent assay (ELISA)**

There are two versions of the BAb ELISA, the indirect ELISA and the sandwich ELISA, also known as capture ELISA.

In the indirect ELISA, the IFN-beta antigen is coated onto a microtiter plate and washed with a wash buffer containing phosphate buffered saline (PBS) and Tween detergent. Sites that have not bound to the antigen are blocked with bovine serum albumin (BSA) and washed. Diluted test serum is then added to the plate. After washing, anti-IFN-beta antibodies are detected with a goat-anti human antibody conjugated to an enzyme which converts a substrate to give off a chromogenic signal that can be quantified in a spectrophotometer. A high optical density is observed in the presence of BAb.

In the sandwich ELISA (or capture ELISA), a capture antibody – a monoclonal mouse anti-human-IFN-beta antibody – is coated onto the plate prior to the addition of IFN antigen to improve the specificity and sensitivity of the assay.

Both assays are mostly qualitative assays. To determine positivity, sera from healthy controls are included in each assay in addition to proper positive and negative controls. The average plus two or three standard deviations of the healthy controls serves as a cut-off for the positivity. Samples with optical densities above this cut-off are considered positive and samples with optical densities below the cut-off point are considered negative.

1.6.2.1.2 **Radioimmunoprecipitation assay (RIPA)**

In RIPA, the IFN-beta antigen is iodinated with $^{125}$I. Radio-labelled $^{125}$I-IFN-beta is incubated with test sera in a phosphate triton X-100 buffer (PTX buffer). Sheep anti-human IgG is added to form a visible precipitate with human sera. The precipitates are centrifuged and washed with PTX buffer and counted on a gamma counter. Precipitates containing BAb would have bound to $^{125}$I-IFN-beta. In precipitates without BAb, $^{125}$I-IFN-beta would have been washed away. The mean value of healthy sera is subtracted from value of the test sample. The amount of radioactivity is proportional to the BAb present in the serum. Results are expressed as a percentage of the value obtained with a known high positive serum (Lawrence, et al., 2003).
1.6.2.2 Measuring anti-interferon-beta neutralizing antibodies
To identify NAbs, a bioassay is used. Generally, patient sera is pre-incubated with IFN-beta then added to a cell line which responds to IFN-beta. Several methods are available to measure neutralizing antibodies to interferon-beta.

1.6.2.2.1 Cytopathic effect (CPE) assay
The cytopathic effect (CPE) assay measures the antiviral activity of IFN-beta in protecting mammalian cells from cell death upon challenge by a virus (Grossberg, et al., 1986). The CPE assay has been viewed as the gold standard for measuring NAbs (Gilli, et al., 2004). In brief, approximately 10 units of IFN-beta is pre-incubated with heat inactivated diluted test sera and added to cells on a microtiter plate. In addition to dilution series of test sera, a dilution series of IFN-beta and controls for serum toxicity are also included in each assay. After 12-24 hours of incubation, the cells are exposed to a virus. Subsequent to another 18-24 hours of incubation, a dye is finally added to quantify IFN-beta’s ability to prevent cell lysis.

Like other NAb assays, NAb titres in the CPE assay are calculated using the Kawade formula and reported in tenfold reduction units (TRU)/ml. Samples with titres under 20TRU/ml are generally accepted as absent for NAb. The tenfold reduction unit describes the dilution of serum required to reduce the activity of 10 units of interferon to 1 unit of interferon. According to Grossberg and colleagues, since NAbs have relatively low affinity, the proportion of antibody bound to IFN-beta is static at any time. As such, the amount of antibody required to reduce IFN-beta activity from 10 units to 1 unit is the same as the amount of antibody required to reduce IFN-beta activity from 30 units to 3 units (Grossberg, et al., 2001a; Grossberg, et al., 2001b). The tenfold reduction unit is recommended by the World Health Organization (WHO) to help account for variations in the sensitivities of the different bioassays used to measure NAbs (Grossberg, et al., 2001a; Grossberg, et al., 2001b).

In the Kawade formula, titre (t) = f (n-1)/9, where t = titre in TRU/ml, f = reciprocal of serum dilution at endpoint, and n = actual amount of IFN antigen in test as lab units (LU)/ml. The endpoint of the CPE assay is at fifty-percent cell death. The concentration of IFN at the endpoint is defined as 1 LU/ml and needs to be determined in each day’s assay. To determine n, the actual amount of IFN antigen used in each day’s test as LU/ml, the reciprocal of the IFN dilution at the end point is divided by the reciprocal of the IFN dilution used during the preincubation step. The reciprocal of the serum dilution required to achieve the end point is designated as f in the Kawade formula.
Fundamentally, the Kawade formula is a cross multiplication step to correct for the unknown amount of IFN (in LU/ml) used during the preincubation step. Since the endpoint of the assay is determined after each day’s assay, the amount of IFN (in LU/ml) used during the preincubation step can only be determined after the assay. If we rearrange the formula, we have \( t/f = (n-1)/(10-1) \).

Many variations of the CPE exist and different combinations of cell lines and viruses have been used and validated, including Wistar Institute Susan Hayflick (WISH) cell line in combination with vesicular stomatitis virus (VSV) (Massart, et al., 2007). The WHO recommends using the lung carcinoma cell line A549 in combination with the encephalomyocarditis (EMC) virus (World Health Organization Expert Committee on Biological Standardization, 1985).

### 1.6.2.2.2 Myxovirus protein A (MxA) assay

In the Myxovirus protein A (MxA) assay, IFN-beta is preincubated with diluted sera and added to A549 cells which respond to IFN by producing the type I IFN specific MxA, an antiviral protein with GTPase activity. MxA protein can be measured using enzyme linked immunosorbent assay (ELISA) (Files, et al., 1998; Pungor, et al., 1998).

Similar to the CPE assay, the Kawade formula and the tenfold reduction unit is used to calculate and report antibody titres in the MxA assay. However, neutralizing units (NU)/ml is often used to designate titres obtained by the MxA assay.

The MxA assay was developed to overcome the shortcomings of antiviral neutralization assays (Files, et al., 1998). First, the MxA assay does not involve cultivating viruses which allows more laboratories to be equipped for performing the assays. Secondly, the MxA assay has fewer steps and requires less time than the CPE assay, which are 2.5 days for MxA assay and 4 days for the CPE assay. However, there are some limitations to the MxA assay as well: the detection antibody for MxA is not widely available.

Variation of the MxA induction assay exists, where mRNA is measured and quantified by reverse transcriptase quantitative real time polymerase chain reaction (RT-qPCR) (Bertolotto, et al., 2007). In addition to MxA mRNA, quantifying type I IFN-inducible 6-16 mRNA using RT-qPCR and branched DNA (bDNA) is also feasible for measuring NAbs to type I IFNs (Moore, et al., 2009).
1.6.2.2.3 Reporter gene assays
In addition to the CPE and MxA assays, reporter gene assays have been developed to measure neutralizing antibodies to IFN-beta. In these assays, the promoter of an ISG is linked to a reporter gene such as luciferase. Several cell lines are available and contain ISG promoters such as ISRE, IFI-161, or the Mx promoter.

1.6.2.3 Cross reactivity of NAbs
Anti-IFN-beta NAb titres are different dependent on the antigen used. This has been observed in the CPE assay as well as the MxA protein assay (Khan and Dhib-Jalbut, 1998; Bertolotto, et al., 2000; Files, et al., 2008). Lower titres are obtained when sera is assayed with IFN-beta-1b than with IFN-beta-1a. Titres assayed on IFN-beta-1a are 3-5 times higher than when assayed on IFN-beta-1b (Files, et al., 2008). It is unknown whether this is a property of the antibodies or a property of the antigen used.

Massart and colleagues took advantage of the cross reactivity of NAbs and used IFN-beta-1a as the antigen to increase the sensitivity of the NAb assay. In a study of 185 patients treated with IFN-beta for more than 1 year (62 Betaseron®, 62 Rebif®, 61 Avonex® treated), NAbs were tested using both antigens. Four patients tested NAb-negative using IFN-beta-1b as the antigen but tested NAb-positive using IFN-beta-1a as the antigen. All four patients were Betaseron®-treated and they became NAb positive 3-6 months later using when using IFN-beta-1b as the antigen (Massart, et al., 2008).

Recently, the European Medicines Agency (EMA) has recommended that all NAb tests be conducted on the IFN-beta-1a antigen (EMA, 13 Feb 2008). This is supported by the fact that IFN-beta-1a shares 100% sequence homology and glycosylation sites with natural human fibroblast IFN and that NAbs that neutralize IFN-beta-1a neutralizes natural human fibroblast interferon in the same manner (Sominanda, et al., 2009).

On the other hand, this has implications on how “high titres” are defined and has impact on how antibody results are interpreted. How NAbs are measured should be clarified in each report and readers need to be aware of these difference in the extensive literature on anti-IFN-beta antibodies.

1.6.3 Measuring interferon-beta biological response
The use of biomarkers for monitoring drug response depends on the knowledge of the action of mechanism of the specific drug in question. The signalling pathways and networks affected by
the drug must be well defined. For IFN-beta, signalling pathways and ISGs affected are well defined, but the exact mechanism of action for IFN-beta is unknown. Several markers have been used to measure the biological response of IFN-beta.

1.6.3.1 Neopterin

Neopterin is a marker associated with Th1 cell activation and cell-mediated immunity. It is a chemical produced by macrophages and monocytes upon stimulation by IFN-gamma from T-cells (Hamerlinck, 1999). Neopterin is not a direct measure of IFN-gamma activity, but reflects the immunological network of interactions on macrophage and monocytes populations. Unlike biologically active cytokines which are locally produced and quickly bound to nearby targets or easily neutralized by soluble receptors, neopterin is a biochemically inert metabolite of guanosine phosphate (GTP), making it a valuable immunodiagnostic tool. Neopterin levels are significantly higher in several autoimmune and neoplastic disease states compared with healthy controls (Hamerlinck, 1999). For MS, the relationship is inconclusive and contradicting results have been reported (Bagnato, et al., 2003).

Neopterin is inducible by IFN-beta and has been used to measure the pharmacodynamic and pharmacologic effects of IFN-beta-1a and IFN-beta-1b (Williams and Witt, 1998; Rudick, et al., 1998; Cook, et al., 2001; Bagnato, et al., 2003; Sorensen, et al., 2006). Concentrations of neopterin peak 24-48h after the first injection of IFN-beta-1a (Bagnato, et al., 2002).

In the open-label extension study of IM IFN-beta-1a, serum neopterin 48h post injection was measured at week 52 of treatment. In NAb positive patients with titres of 5 and above, in vivo neopterin induction was progressively attenuated and highly significant at titres above 20. Neopterin levels directly correlated with the titre of NAb (Rudick, et al., 1998). Cook and colleagues also reported that mean serum neopterin levels were lower in patients with high (>60) as compared to low NAb titres (Cook, et al., 2001).

1.6.3.2 Beta-2-microglobulin

Beta-2-microglobulin (B2M) is subunit of MHC class I, which is found on nearly all nucleated cells and present antigenic peptides to cytotoxic CD8+ T cells. All IFN family members share the ability to enhance the expression of MHC Class I proteins to enhance the CD8+ T cell response and can be used as a marker for IFN response (Stark, et al., 1998). B2M has also been used as a marker for renal function and cancer.
B2M is non-covalently linked to the alpha chain of the MHC molecule and exchange freely with soluble B2M (Bjorkman and Burmeister, 1994). Under normal physiological conditions, B2M protein is present at low levels in serum and urine and is almost exclusively catabolised in the kidney (Shi, et al., 2009). B2M has an approximate molecular weight of 11,000 Da and is considered a middle-sized molecule. It accumulates in patients with chronic renal failure and can be used to assess renal function. In patients on long-term haemodialysis, it can aggregate into amyloid fibrils and deposit into osteo-articular tissues and result in dialysis-related amyloidosis (Drüeke and Massy, 2009).

Other than its role in immunity, B2M is also involved in the regulation of survival, proliferation, apoptosis and metastasis in cancer cells. Free B2M in urine or serum is regarded as an independent prognostic marker in several cancers and is emerging as a potential therapeutic target in cancer (Shi, et al., 2009).

As a well-established ISG, B2M has been used as a pharmacodynamic marker for IFN-beta response (Rothuizen, et al., 1999).

Sorensen and colleagues investigated the effects of NAb on IFN-beta bioactivity as measured by neopterin and beta-2-microglobulin levels on thirty-two patients who were treated with IFN-beta. NAbs were measured by CPE and categorized according to the neutralizing capacity (NC) where NC<20% was defined as negative NAb, NC between 20 to 79% was defined as low NAb was and NC>80% was defined as high NAb. All NAb negative patients showed elevated levels of beta-2-microglobulin and or neopterin, whereas all high-positive patients had no biological response. Patients with both neopterin and beta-2-microglobulin response had less MRI activity than patients without biological response (Sorensen, et al., 2006). The absolute levels of neopterin and B2M were reduced in a titre-dependent fashion in patients who were NAb positive (Scagnolari, et al., 2007).

1.6.3.3 Matrix metalloproteinases, tissue Inhibitors, and intercellular adhesion molecules

Matrix metalloproteinases (MMPs) has a role in mediating the blood brain barrier and the extravasation of immune cells into the brain. Several lines of evidence have shown that elevated levels of MMPs, in particular MMP-9, are found in brain tissue, serum and CSF from patients with MS (reviewed by Yong, et al., 2007). A proposed mechanism of action for IFN-beta is the improvement of blood-brain barrier integrity partially through a reduction of MMPs, an increase of its tissue inhibitors (TIMPs) and an increase in serum levels of intercellular adhesion

Trojano and colleagues reported that there was a decrease in serum MMP-9 during the first three months of IFN-beta therapy which was sustained during the first 18 months of treatment (Trojano, et al., 1999). Galboiz and colleagues also showed that there was a suppression of MMP-9 in PBMC of patients treated with IFN-beta (Galboiz, et al., 2001). Karabudak and colleagues reported that MMP-9 levels did not change during a one year period with IFN-beta therapy, but levels of TIMP-1 increased (Karabudak, et al., 2004). Conversely, Bernal and colleagues reported that MMP-9 responded to IFN-beta therapy but TIMP-1 remained unchanged over the first six months of IFN-beta therapy (Bernal, et al., 2009). Boz and colleagues reported that serum MMP-9 and MMP-9/TIMP-1 ratio decreased over six months of IFN-beta therapy. Although changes in serum TIMP-1 was not statistically significant, CSF levels of TIMP-1 increased after six months of therapy (Boz, et al., 2006). Avolio showed that serum MMP-9/TIMP-1 ratio decreased during the first 48 months of IFN-beta treatment and that it may be predicative of MRI activity in RRMS (Avolio, et al., 2005). A two-fold decrease in MMP-9/TMIP-1 ratio by quantitative RT-PCR was suggested as a marker for high IFN-beta bioavailability (Garcia-Montojo, et al., 2010).

With regards to neutralizing antibodies, MMP-9 levels tended to be higher in NAb positive patients (Trojano, et al., 1999). Gilli and colleagues found that the presence of NAbs reversed the effects of IFN-beta therapy down-regulated expression of MMP-9 in PBMC of patients who have been treated for more than three months. Moreover, MMP-9 expression showed a quantitative correlation with NAb titre whereas MxA expression levels did not (Gilli, et al., 2004).

In contrast, Comabella and colleagues reported that the presence of NAbs had no effect on MMPs or TIMPs levels but an increase in TIMP-1 levels correlated with a good clinical response to IFN-beta regardless of NAb status in 43 RRMS patients (Comabella, et al., 2009).

1.6.3.4 **TNF-related apoptosis inducing ligand (TRAIL)**

TNF-related apoptosis inducing ligand (TRAIL) has a role in immunoregulatory processes such as apoptosis (Chawla-Sarkar, et al., 2001) and inhibition of T cells (Wandinger, et al., 2003, Song, et al., 2000). It is an IFN-beta inducible product and is upregulated during IFN-beta treatment (Arbour, et al., 2005). TRAIL has been investigated as a prognostic marker for IFN-beta clinical treatment response as well as a marker for detecting the loss of IFN-beta biological activity due to NAbs.
Wandinger and colleagues reported that early sustained TRAIL expression could distinguish IFN-beta responders from non-responders, with responders as defined by patients without relapses during the first year of IFN-beta treatment. In NAb positive patients, the initial TRAIL upregulation was abrogated. The authors also found that pre-treatment serum soluble TRAIL was higher in responders and may be predictive of treatment response (Wandinger, et al., 2003). On the other hand, in a prospective study of 30 patients followed by blinded investigators, TRAIL only correlated with flu-like adverse effects of IFN-beta, but did not predict one year or two year clinical treatment response (Buttmann, et al., 2007).

TRAIL mRNA expression can detect lost IFN-beta bioactivity due to NAbs, although for this purpose, TRAIL mRNA was less sensitive than MxA mRNA (Gilli, et al., 2006; Santos, et al., 2006; Sominanda, et al., 2008). The abrogation of TRAIL mRNA in NAb-positive patients was titre-dependent (Sominanda, et al., 2008).

1.6.3.5 Myxovirus protein A
MxA is an IFN-beta inducible product specific to type I interferons. Although it is an antiviral GTPase that is unlikely related to the beneficial effects of the IFN-beta in MS, it is the most commonly used biomarker for IFN-beta bioactivity.

1.6.3.5.1 MxA protein as a biomarker
Deisenhammer and colleagues described the use of MxA protein as a biomarker of IFN-beta biological response and showed that whole blood MxA protein was significant lower in NAb positive patients compared to NAb negative patients (Deisenhammer, et al., 1999). Vallittu and colleagues also showed that MxA protein is a suitable marker for identifying non-responding patients and moreover it negatively correlates with NAb titre (Vallittu, et al., 2002). Nonetheless, MxA protein is not as frequently used as MxA mRNA because of the limited availability of commercialized antibodies to use in assays to measure the MxA protein.

1.6.3.5.2 MxA mRNA as a biomarker
Measuring MxA mRNA as a biological marker for IFN-beta biological response was introduced by Bertolotto’s group in 2001. RNA extracted from peripheral blood mononuclear cells (PBMCs) was semi-quantified using a quasi quantitative polymerase chain reaction (PCR). Unsurprisingly, MxA mRNA levels were lower in NAb positive patients when compared to NAb negative patients (Bertolotto, et al., 2001). Pachner’s group used real time quantitative PCR (RT-qPCR) to quantify MxA in whole blood collected in PAXgene™ tubes to improve the
isolation of RNA, allowing the immediate stabilization of RNA upon phlebotomy, minimizing post-phlebotomy blood processing variables (Pachner, et al., 2003).

In a recent study, Hesse and colleagues showed that no bioactivity is preserved in NAb positive patients when there is an absence of MxA induction (Hesse, et al., 2009). In this study, gene expression was measured by gene chip analysis where 8793 genes were screened. 1,077 IFN-beta regulated genes were identified in NAb-negative patients, none of which were detected in NAb positive patients. The authors suggest that in NAb positive patients with a lack of in vivo MxA response, IFN-beta biological response is completely abolished with no residual bioactivity.

1.6.3.5.2.1 Timing of blood draws for MxA mRNA measurements
MxA mRNA rises and declines after an injection. One of the benefits of using MxA mRNA compared to MxA protein is its short half life. Because of its shorter half life, one ascertain that the presence of MxA mRNA is due to IFN-beta injections, rather than subclinical infections which can increase IFN-alpha and thus MxA. Most studies use 9-12 hours or 12 hours post IFN injection as the time of blood draw for measuring MxA mRNA (Bertolotto, et al., 2001; Bertolotto, et al., 2003; Pachner, et al., 2003; Gilli, et al., 2004a, Gilli, et al., 2004b; Bertolotto, et al., 2004; Pachner, et. al., 2005; Pachner, et al., 2006; Gilli, et al., 2006; Capra, et al., 2007; Sominanda, et al., 2008; Malucchi, et al., 2008; Hesse, et al., 2009a, Hesse, et al., 2009b; Ravnborg, et al., 2009; Pachner, et al., 2009a; Pachner, et al., 2009b). However, this is debatable as others have found that MxA peaked as early as 3-4 hours post IFN injection (Santos, et al., 2006; Millonig, et al., 2009).

1.6.3.6 Clinical relevance of measuring the biological response to interferon-beta using MxA mRNA
Compared to NAbs, MxA had a slightly stronger prognostic significance in predicting the risk of new relapses. (Malucchi, et al., 2008) and a lack of IFN-beta bioactivity is associated with the occurrence of relapses (van der Voort, et al., 2009b). Van der Voort’s group suggests that MxA expression can be used as a screen test to identify non-responders (van der Voort, et al., 2009a).
1.7 Rationale and objectives

1.7.1 Luciferase reporter gene assay to measuring neutralizing antibodies to interferon-beta in multiple sclerosis

Current methods to measure anti-IFN-beta antibodies are time consuming and require multiple steps which may be cumbersome. The CPE assay requires 4 days and the MxA assay requires 2.5 days. There is an unmet need for NAb assays which are easy to perform without compromising assay performance. Our objective is to develop a rapid, easy to perform NAb assay which is specific and sensitive.

1.7.2 Real-time polymerase chain reaction of MxA mRNA to measure the bioavailability of interferon-beta

Measurement of MxA mRNA expression can be used to evaluate antibody mediated decreased bioactivity of IFN-beta. Compared to NAb, this method may be a more direct method in evaluating drug efficiency. MxA mRNA levels rise and decline upon administration of the drug. The timing of blood draw is an important parameter for MxA mRNA measurements and a critical consideration in standardizing the MxA bioavailability test. This has been debated and the optimal time to draw blood remains unclear. Early studies indicate that 12h after injection is the optimal time point for collecting blood mRNA (Pachner, et al., 2003), but recent studies have shown that MxA mRNA levels are elevated as early as 3-4 hours after IFN administration (Santos, et al., 2006; Gilli, et al., 2006). Our objective is to determine the optimal sampling time which shows the most robust MxA mRNA expression to best assess drug bioavailability. Additionally, we would like to study how NAbs affect the biological activity of IFN-beta and at what titre, as measured by the luciferase reporter gene assay, is the biological activity lost.
1.8 References


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Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFN-beta in multiple sclerosis patients

2.1 Introduction

Interferon beta (IFN-beta) is a first line treatment for multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system (CNS) characterized by inflammatory demyelination. Therapy with IFN-beta reduces disease exacerbations and magnetic resonance imaging (MRI) burden (The IFN Beta Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group, 1996; Giovannoni, et al., 2002). However, in a proportion of patients on therapy, binding antibodies (BAbs) and neutralizing antibodies (NAbs) occur. NAbs reduce bioavailability of IFN-beta to its receptors and high NAb titres reduce the efficacy of the drug. NAb positive patients may have higher relapse rates (Boz, et al., 2007), more disease progression and more new or enlarging MRI lesions in comparison to NAb negative patients (Francis et al., 2005).

BAbs and NAbs are defined by the assay method used. BAbs are measured by binding assays such as enzyme linked immunosorbent assays (ELISA), radioimmunoprecipitation assays (RIPA), or column chromatography assays, which determine the presence of antibodies that bind to IFN-beta. NAbs are defined by inhibition of in vitro bioassays that measure the activity of IFN-beta in the presence of sera containing NAbs. Two common assays are available to measure NAbs. The cytopathic effect (CPE) assay measures the antiviral activity of IFN-beta upon challenge of a cell line with a virus. The myxovirus resistance protein A (MxA) induction assay measures IFN-beta inducible gene product MxA protein or MxA mRNA to quantify IFN-beta response (Files, et al., 1998; Pungor, et al., 1998; Bertolotto et al., 2007). Because these methods of NAb detection are arduous, a BAb assay can be used to screen for further NAb testing (Sorensen, et al., 2005). There is a need for a new, specific, sensitive and less arduous assays for quantifying NAbs in IFN-beta treated MS patients. Recently, reporter gene assays have been described to measure NAbs (Farrell, et al., 2008 and Lallemand, et al., 2008).

We report the validation of a luciferase reporter gene assay to detect NAbs based on the activation of the early IFN inducible 6-16 promoter by IFN, described by R. Farrell and G. Giovannoni (Farrell, et al., 2008). This method uses the luminescence signal from cell line HL 116 which is the human fibrosarcoma cell line HT1080 transfected with a plasmid containing a luciferase cDNA controlled by the immediate early IFN inducible 6–16 promoter (Uze, et al., 1994). 6–16 is a gene specific only to type I interferons (Der, et al., 1998). When IFN-beta binds to receptor, luciferase is produced and the response to IFN-beta can be quantified by a
luminometer upon the addition of substrate. Patients' sera are serially diluted and incubated with a constant amount of IFN-beta (10U/ml). In the presence of NAbs, less luciferase is produced. Results are reported in Tenfold Reduction Units (TRU)/ml (Grossberg, et al., 2001) as per WHO recommendations. We validated this assay by comparing NAb status and NAb titres obtained on split samples with the reference A549/EMCV CPE considered gold standard (Sorensen, et al., 2005) assayed at the Medical College of Wisconsin (Grossberg, et al., 1986).

2.2 Methods

2.2.1 Sera
Sera were selected from the University of British Columbia MS serum bank on the following criteria; a) treated with a single IFN-beta product, b) availability of antibody results from the routine BAb testing by the sandwich ELISA and when positive were further tested with the A549/EMCV CPE method by S. Grossberg (Grossberg, et al., 1986). Sera were collected more than 12 hours after the last interferon injection, a time when circulating levels of injected interferon are below threshold detectable by bioassay.

A total of 163 sera were tested for the presence of NAbs using the luciferase reporter gene assay as well as the sandwich ELISA and A549/EMCV CPE assays. Of 163 samples, 82 samples were from patients treated with IFN-beta-1b (Betaseron®), 81 from patients treated with IFN-beta-1a (Rebif®). Samples were chosen to represent a range of NAb titres from negative to high positive. Samples were then blinded before being assayed.

2.2.2 NAb testing by luciferase reporter gene assay

2.2.2.1 Cell lines, media, growth conditions
Cell line HL116 (a gift of Dr. G. Uzé, France) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM sodium hypoxanthine, 0.4 µM aminopterin, 0.016 mM thymidine, 100U/ml penicillin G, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B (culture medium) at 37 °C, 5% CO₂. Cells were passed twice weekly at a 1:8 dilution with a limit of 25 passages. During the assay, cells were grown in culture medium without antibiotics and antifungotics.

2.2.2.2 Bioassay parameter testing
Several parameters of the luciferase assay were studied. We studied the effect of plate color, plating cell density, plate incubation time, amount of substrate used, duration of plate shaking,
and methods of drawing dilution curves in order to optimize the assay procedure (Gupta, et al., 2007; Lai, et al., 2006).

2.2.2.3 Optimized bioassay procedure
Patient’s sera were heat inactivated for 30 min at 56 °C then stored at 4 °C for up to 2 weeks until assayed. Cultured HL116 cells were visually inspected for viability and seeded at 40,000 cells/well in 96-well black plates with a transparent bottom (Costar 3603, NY, USA) and incubated for 16 h at 37 °C, 5% CO2. Serum samples were serially diluted and pre-incubated with interferon for 1 h then added to the microtiter plates at 100 µl/well, discarding overnight culture media. After 5 h incubation at 37 °C, plates were cooled to room temperature (10 min), the plate bottom was sealed with backing tape (PerkinElmer 6005199, USA) to prevent light contamination and 50 µL Steadylite® substrate (PerkinElmer, USA) was added. Luminescence signal was immediately read (Wallac VICTOR®Light 1420 Luminescence Counter, PerkinElmer, USA) after 1 min of shaking.

We used the luciferase assay as both a NAb screening and NAb titre assay. For the NAb screening assay, 1:10 and 1:500 diluted sera were added to an equal volume of the corresponding IFN-beta, IFN-beta-1b (Berlex, CA, USA) or IFN-beta-1a (Serono, Canada; Biogen Idec, Canada) at a concentration of 20LU/ml. Positive samples were re-assayed in the NAb titre assay, where sera were diluted 1:2 fold serially from an initial dilution of 1:10, 1:100 or 1:500 and added to an equal volume of IFN-beta at 20LU/ml, six dilutions were made for each test sera. For quality control, at least two points must be above and below the 50% cut off for the serum dilution to be valid. In parallel, a homologous interferon (IFN) dilution curve was included on each plate to determine the activity of the antigen used. We defined one Laboratory Unit (LU) as the end-point of our assay at 50% luminescence signal for the interferon dilution curve on a log–log scale. Only assays in which the challenge dose of IFN-beta was between 7–15 LU/ml were accepted. Positives were determined by signals below the < 50% cut-off (> 20TRU/ml). Positive samples were serially diluted and reassayed. Titres were calculated with the Kawade–Grossberg Formula $t = f^*(n - 1)/9$, where $t$ = titre in Ten Fold Reduction Units (TRU)/ml, $f$ = reciprocal of serum dilution at end-point, $n$ = actual amount of IFN antigen in test as LU/ml.

2.2.3 NAb testing by ELISA/CPE
NAb testing by ELISA and CPE have been previously described (Boz, et al., 2007). A sandwich ELISA was used to test for BAbs as a screening assay for further NAb testing by the
A549/EMCV CPE method. BAb+ samples were sent to the Medical College of Wisconsin for assay by the cytopathic effect (CPE) assay using A549 human lung carcinoma cells and the encephalomyocarditis virus (Grossberg, et al., 1986). Titres were calculated by the Kawade–Grossberg Formula and reported in Ten Fold Reduction Units (TRU)/ml. Samples were considered negative if they were BAb negative by ELISA or if they had titres lower than 20TRU/ml for the CPE assay.

2.2.4 Statistical analysis

The Fisher's Exact Test and Kappa inter-rater agreement was used to compare the NAb frequencies obtained with the two methods to measure NAbs. Positive NAb titres found by the luciferase assay and by the CPE assay were compared with the Wilcoxon matched-pairs signed rank test. Linear regression analysis was performed to determine the correlation of NAb titres.

2.3 Results

2.3.1 Characterization and optimization of the luciferase assay

2.3.1.1 Plate color

Three types of sterile, tissue culture plates were tested for use in the luciferase assay: 96 well white plates with a transparent bottom (6005181, PerkinElmer, USA), black plates with a transparent bottom (Costar 3603, NY, USA), and black plates with a white bottom (6005688, PerkinElmer, USA). White plates yielded higher counts, but light contamination between neighbouring wells was observed. Higher counts were observed for the black plates with the white bottom, on a log–log transformation, there was no difference in the characteristics of the IFN dilution curve between the two black plates and there was no effect on assay sensitivity to IFN. Black plates with a transparent bottom were chosen, which also allowed the monitoring of cell viability prior to the assay.

2.3.1.2 Plating cell density

To determine the optimal number of cells plated, yielding low background and maximal IFN response, the effect of cell density was evaluated by seeding 100, 500, 1000, 5000, 10 000, 20 000, 40 000 cells per well in 96 well plates. On a log–log scale, there were no significant difference in maximum/minimum response for the different number of cells seeded, but the coefficient of variation was < 0.01 for triplicate wells when 40,000 cells/well were
seeded, hence 40,000 cell/well was chosen for our assay, providing optimal cell response with minimal cell death. (Figure 2.1)

2.3.1.3 Interferon exposure time
IFN-induced expression of luciferase in HL116 cell line was measured after 2 h and 5 h of IFN exposure at 37 °C to determine the optimal cell response. Five h of IFN exposure time yielded maximal IFN response and less well-to-well variation in triplicate wells versus 2 h IFN exposure.

2.3.1.4 Amount of luciferase substrate
To quantify the amount of IFN-induced luciferase, the amount of Steadylite® substrate added was varied from 100 µl (manufacturer's recommendation) to 50 µl, and 25 µl. There was no difference between 100 µl or 50 µl of added substrate. Cell response was limited when only 25 µl of substrate was used. Fifty µl of substrate was chosen to minimize assay cost.

2.3.1.5 Plate shaking
Upon addition of substrate, plates were shaken in the luminescence counter to ensure that cells were lysed. We tested 1, 5, or 10 min of plate shaking and observed no difference in the interferon dilution curve. 1 min of plate shaking was chosen to minimize variability in IFN exposure time when multiple plates are assayed on the same day.

2.3.1.6 The interferon dilution curve
With the optimized parameters, luminescence readouts (counts per second) given by the luminescence counter was highly variable at high IFN concentrations. This may be due to the inherent properties of the assay system used. When log (counts per second) were used, we were consistently able to generate IFN dilution curves that had the same slope, delta, and half effective maximal concentration (EC50) using a four-parameter curve fit.

2.3.2 Assay sensitivity to interferon beta: dose-dependent luciferase activity induced by interferon-beta in HL116 cells
Using the optimized assay procedure, the sensitivity of this assay to IFN-beta-1b and IFN-beta-1a was evaluated using serial dilutions of NIH standard Gxb02-901-535 (rHu-IFN-beta-1b) and Gb23-902-531 (rHu-IFN-beta-1a). Luciferase activity was measured after 5 h of IFN exposure. Media alone induced minimal luciferase activity. On a log–log scale, the IFN curve is linear from 1 to 25 IU/ml using NIH standard which corresponds to 0.2 LU/ml to 5 LU/ml when one Laboratory Unity is defined as half of maximal luciferase activity. Hence, 1 LU/ml of IFN in our
assay corresponds to 4 IU/ml of WHO reference reagents (Figure 2.2). The dose response curves of IFN-beta-1b and IFN-beta-1a were superimposable to NIH standards.

2.3.3 WHO international reference antiserum

The titre for the WHO International Reference Reagent G038-501-572 (human anti human IFN-beta antiserum) was evaluated using the luciferase assay. The log10 titre obtained in the luciferase assay is similar to that obtained by the WHO International Collaborative Assay Study Group I (Grossberg, et al., 2001). The results are tabulated in Table 2.1. (Table 2.1) (Figure 2.3). The antibody blocked IFN-beta-1b to a level of one third that of IFN-beta-1a.

2.3.4 Inter-assay variability and intra-assay variability

Inter-assay and intra-assay variation were evaluated by assaying five pooled sera as in-house controls. These sera were selected from the UBC MS serum bank and have been previously assayed by CPE and classified to have low or high positive titres for each of the corresponding interferons.

Inter-assay variability was evaluated by repeating the five pooled sera on separate days, 7, 16, 12, 11, or 11 times respectively. The coefficient of variation for the inter-assay variability was between 17.8% to 29.3% (21.3%, 24.8%, 17.8%, 29.3%, 24.1%, respectively) (Table 2.2). Intra-assay variability was evaluated by assaying the five pooled sera on the same day 3, 3, 3, 2 or 3 times, respectively, by different operators. The coefficient of variation for the intra-assay variability was between 6.3 to 15.2% (15.2%, 14.5%, 6.3%, 12.9% or 7.3%, respectively) (Table 2.3). These variations are acceptable for a biological test, especially considering the great variability of NAb assays reported (Sorensen, et al., 2005).

2.3.5 Correlation of NAb titres measured by luciferase vs. CPE

We compared the results obtained with the luciferase assay system to those obtained by ELISA as a screening followed by titration with CPE on blinded paired samples. Out of 163 samples, we found 7 discordant samples. The frequency of NAbS detected by luciferase and the sandwich ELISA/CPE assay was compared in contingency tables (Table 2.4 and Table 2.5). The two tailed p value for Fisher’s exact test was < 0.0001 for both IFN-beta-1b and IFN-beta-1a. The kappa agreement is 0.838 for IFN-beta-1b and 0.966 for IFN-beta-1a. There is no significant difference between NAb status as determined by the luciferase system or ELISA/CPE methods. Samples positive with luciferase and negative with CPE all had low titres: 21, 26, 50, 64, 70 TRU/ml (IFN-beta-1b) and 49 TRU/ml (IFN-beta-1a). Conversely, the single
false negative sample was < 20 TRU/ml by luciferase and 21 TRU/ml by CPE. Twenty TRUs/ml is considered to be the lowest limit for antibody positivity.

The titres obtained from the CPE assay had a median of 212 TRU/ml, range 21 TRU/ml to 8246 TRU/ml for IFN-beta-1b and a median of 772 TRU/ml, range 32 TRU/ml to 41040 TRU/ml for IFN-beta-1a. The titres obtained from the luciferase assay has a median of 272 TRU/ml, range 20 TRU/ml to 7743 TRU/ml for IFN-beta-1b and a median of 1078 TRU/ml, range 45 TRU/ml to 20586 TRU/ml for IFN-beta-1a. NAb titres of positive samples were evaluated using the Wilcoxon signed rank test. There was no significant difference between the luciferase and the CPE methods used to measure NAbs (Z = −375; p = 0.071 for anti-IFN-beta-1b and Z = 387; p = 0.165 for anti-IFN-beta-1a). Log10 NAb titres correlated very well with the two methods (R2 = 0.860; p < 0.0001 for for IFN-beta-1b and 0.936; p < 0.0001 for IFN-beta-1a) (Figure 2.4). These results demonstrate that the luciferase and the CPE assay (considered the gold standard) generate very similar results.

2.4 Discussion

In this study the R. Farrell and G. Giovannoni luciferase assay methodology was validated for the detection of NAbs to IFN-beta. We found that the parameters that optimize assay sensitivity and reproducibility include using a 96-well black plate with a transparent bottom allowing the visual inspection of cells, seeding 40,000 cells/well allowing high sensitivity to IFN-beta with low coefficients of variation between wells, incubating plates 5 h permitting a one-day rapid assay without compromising assay sensitivity, 50 µl of luciferase substrate minimizing cost, and 1 min of plate shaking minimizing between plate variability. Log transformation of the luminescence readouts (obtained in counts per second) ensures the reproducibility of the interferon dilution curve by reducing the variability of luciferase activity at high IFN-beta concentrations. By defining 1 LU/ml at 50% luminescence signal on a log–log scale, the interferon dilution curve in the luciferase system is similar to that of the CPE system, where IFN-induced activity is linear from 0.2 to 5 LU/ml.

Although it has been suggested that IFN-beta-1a be used as a single target antigen for all samples tested no matter which IFN-B preparation the patient has received (European Medicines Agency, 2008), we used use a homologous system where the target antigen used corresponds to patient therapy. Others have observed higher NAb titres when IFN-beta-1a is used as antigen in NAb assays (Bertolotto, et al., 2000; Files, et al., 2007). This phenomenon is also observed in our assay; titres 1.5 to 6 times higher were obtained with IFN-beta-1a.
versus IFN-beta-1b as the antigen regardless of patient therapy (unpublished data). This may be due to a difference in the epitope specificity of neutralizing antibodies against IFN-beta-1a or IFN-beta-1b, or is due to denaturation, renaturation or aggregation of IFN-beta-1b. It will be essential to correlate NAb titres to clinical outcomes or in vivo biological markers to validate the use of 20 TRU/ml as a cut-off for NAb positivity in the luciferase assay.

In conclusion, the validation studies described here on the luciferase reported gene bioassay demonstrate the comparability of the IFNB NAb titres it generates and those obtained with the antiviral CPE bioassay. The relative ease of performance of the luciferase assay may allow its use as a qualitative as well as a quantitative NAb assay, eliminating the need for the BAb assay screening test. The assay is reliable, appropriately sensitive and more rapid than currently available NAb methods.

### 2.5 Acknowledgements

We thank Dr. G. Uzé (Montpellier, France) for the generous gift of the cell line transfected with the reporter gene HL116. This study was supported by grants-in-aid from Bayer, Biogen Idec, EMD Serono and Teva Neurosciences and by the Christopher Foundation (Vancouver).
Figure 2.1. Dose-dependent luciferase activity induced by IFN-beta-1a (Rebif) in HL116 cells seeded in 96 well microtiter plates with varying cell densities. Averaged data of triplicate wells. Error bars represent one standard deviation.
Figure 2.2. Dose-dependent luciferase activity induced by IFN-beta in HL116 cells. Averaged data of 3 experiments (IFN-beta-1b) or 6 experiments, (for IFN-beta-1a). Error bars represent one standard deviation.
Figure 2.3. Serum dilution curve obtained from 1:2 fold serial dilutions of WHO International Reference human anti-huIFN-beta antiserum (G038-501-572). Diamond, IFN-beta-1b (Betaseron®) as antigen and ligand; square, IFN-beta-1a (Rebif®) as antigen and ligand; triangle, IFN-beta-1a (Avonex®) as antigen and ligand. Data representative of four individual experiments.
Figure 2.4. Correlation between the luciferase assay and the A549/EMCV CPE assay for log10 NAb titres for IFN-beta-1b and IFN-beta-1a treated patients.
Table 2.1.

Log10 titre of WHO International Reference Human anti Human IFN-beta antiserum (G038-501-572) evaluated using the luciferase assay.*NIH Research Reference Reagent Note 45

<table>
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<tr>
<th></th>
<th>Luciferase Results (log10 titre ± SD), n = 4</th>
<th>WHO International Collaborative Assay Study Group I* (log10 titre ± SD)</th>
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<tr>
<td>Anti IFN-beta-1b</td>
<td>2.80 ± 0.17</td>
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<tr>
<td>Anti IFN-beta-1a</td>
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<tr>
<td>(Rebif®)</td>
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<tr>
<td>Anti IFN-beta-1a</td>
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<td>3.24 ± 0.34</td>
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<td>(Avonex®)</td>
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Table 2.2.

Inter-assay variation for 5 pools of sera

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<th>Treatment</th>
<th>Sample</th>
<th>Average titre</th>
<th>SD</th>
<th>% CV</th>
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<tr>
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<tr>
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<td>196</td>
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<tr>
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<tr>
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<td>39</td>
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Table 2.3.

Intra-assay variation for 5 pools of sera

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample</th>
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<th>SD</th>
<th>% CV</th>
<th>n</th>
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<tbody>
<tr>
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<tr>
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<td>3</td>
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<tr>
<td>Rebif®</td>
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Table 2.4.

Number of NAb positive samples versus CPE/ELISA for IFN-beta-1a treated patients

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</thead>
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<tr>
<td>luciferase +</td>
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<tr>
<td>luciferase -</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>20</td>
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</tbody>
</table>
Table 2.5.

Number of NAb positive samples versus CPE/ELISA for IFN-beta-1b treated patients

<table>
<thead>
<tr>
<th></th>
<th>CPE +</th>
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</thead>
<tbody>
<tr>
<td>luciferase +</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>luciferase −</td>
<td>1</td>
<td>25</td>
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2.6 References


Measurement of interferon-beta bioavailability in interferon-beta treated MS patients using MxA mRNA
3.1 Introduction

Interferon-beta (IFN-beta) is a first line treatment for multiple sclerosis. The disease-modifying drug reduces relapse rates and reduces relapse severity (The IFNB Multiple Sclerosis Study Group, 1993). The development of neutralizing antibodies (NAbs) to IFN-beta occurs in 2-47% of treated patients and reduces drug bioavailability (reviewed by Farrell and Giovannoni, 2007). Factors affecting immunogenicity include the route and frequency of administration, drug formulation, treatment duration, and drug dose (Ross, et. al., 2000). Several methods are available to measure NAbs and these either measure the antiviral effect of IFN-beta (cytopathic effect assay; CPE) or quantify IFN-beta induced products (MxA induction assay, reporter gene assays). NAb measurements are aimed to guide clinicians in determining treatment efficiency; however, it is much debated at which antibody level IFN bioactivity is completely abolished. Furthermore, it is difficult to compare NAb titres between laboratories. Hence, bioavailability measurements become especially valuable in patients with low to moderate NAb titres, “the grey zone” and have been identified as a possible substitute for NAb measurements by NABINMS (Polman, et al., 2010).

The administration of IFN-beta induces an array of IFN-inducible products which regulate the antiproliferative, antiviral and immunomodulatory properties of the drug and the measurement of these products can be used to monitor drug bioavailability. Such markers include the non-IFN-specific beta-2-microglobulin and neopterin and as well as IFN-specific markers such as 2’-5’ oligoadenylate synthetase (OAS), and Myxovirus (Mx) proteins (MxA and MxB) (Rudick, et al., 1998; Stark, et al., 1998; Bertolotto, et al., 2001; Pachner, et al., 2003). Of these markers, the quantification of MxA has been best characterized. Namely, Hesse and colleagues showed that no bioactivity is preserved in NAb positive patients when there is an absence of MxA induction (Hesse, et al., 2009). In NAb positive patients with a lack of in vivo MxA response, IFN-beta biological response is completely abolished with no residual bioactivity.

MxA is an IFN-inducible gene specific for type I IFNs. MxA mRNA levels rise and decline upon administration of the drug. The timing of blood draw is a critical parameter for MxA mRNA measurements and an important consideration in standardizing the MxA bioavailability test. Additionally, two types of measurements have been used to measure bioavailability. MxA mRNA induction refers to the relative increase of MxA mRNA post-injection compared to MxA mRNA levels pre-injection. MxA mRNA expression refers to the absolute amount of MxA mRNA
present at a time post IFN injection. In the latter, a pre-injection sample is not needed, which is more feasible if the test were to be used clinically.

Early studies using MxA mRNA as a biomarker for IFN bioactivity indicated that 12h after injection is the optimal time point for collecting blood mRNA (Pachner, et al., 2003; Pachner, et al., 2005), but recent studies (Table 3.1) have shown that MxA mRNA levels are elevated as early as 3-4 hours after IFN administration (Santos, et al., 2006; Gilli, et al., 2006; Reder, et al., 2008; Millonig, et al., 2009).

In the present study, we aim to determine the optimal sampling time which shows the most robust MxA mRNA expression to best assess drug bioavailability.

### 3.2 Methods

#### 3.2.1 Patients

A total of fourteen IFN-beta treated MS patients participated in this study. The one inclusion criterion was that they were receiving IFN-beta therapy. Subjects were not selected for characteristics of their MS and were recruited by neurologists at the UBC MS Clinic during their routine follow-up visits. Fifteen healthy individuals without medical illness were included as non-injected controls. Informed consent was obtained from all individuals who participated in the study and this study was approved by the University of British Columbia Clinical Research Ethics Board.

#### 3.2.1.1 The effect of drug washout on MxA expression levels

To determine the effect of drug washout on MxA expression, we recruited one NAb negative patient to have blood drawn at the clinic at pre and 2, 4, 6h post IFN injection after a 48h drug washout, then again at pre and 2, 4, 6h post IFN injection after a 7-day drug washout.

#### 3.2.1.2 MxA kinetics in NAb negative patients

To study the dynamics of MxA mRNA in IFN-treated individuals, we recruited NAb negative patients to this study (n=9). Patients were scheduled to have their blood drawn at the UBC MS Clinic on two separate days. On the first day, a pre-injection sample was drawn before IFN-injection, at 2h, 4h, 6h and 8h post IFN. On the second visit, the patient will have taken his or her injection the preceding evening, and arrived at the clinic to have blood drawn at 10h, 12h, 14h, 16h and 18h post IFN. Altogether, blood was drawn before an IFN injection, and every 2h up to 18h post injection. The two visits were no more than 2 weeks apart. A drug washout of 48
hours was included before each IFN injection. Of the 9 patients, 3 were receiving IFN-beta-1b 250μg subcutaneously (SC) every other day (Betaseron®, Bayer, Canada), 4 were receiving IFN-beta-1a 44μg SC three times weekly (Rebif®, Serono, Canada), and 1 was receiving IFN-beta-1a 30μg intramuscularly (IM) once weekly.

3.2.1.3 MxA mRNA in NAb positive patients
To study the effect of NAb on IFN-beta bioavailability, five NAb positive MS patients participated in the study. Patients were eligible if they were NAb positive at the time of study. Blood was drawn pre IFN injection as well as 6h post IFN injection as determined in the MxA kinetics study. A drug washout of 48 hours was included before the IFN injection. Of the 5 patients, 3 were receiving Betaseron®, 1 was receiving Rebif®, and 1 was receiving Avonex®.

3.2.2 MxA mRNA measurements
3.2.2.1 RNA isolation
Whole blood was drawn into PAXgene™ tubes (172165, Becton-Dickinson, Canada) and stored at room temperature for 2 hours to allow reaction with the RNA stabilizing additive, then stored at -20°C until use. Stabilized whole blood RNA was extracted according to the manufacturer’s protocol using the PAXgene™ Blood RNA Kit (762164, Becton-Dickinson, Canada). Extracted RNA was stored at -70°C until use. All samples obtained from one subject were processed in a single batch of RNA isolation. The average RNA yield was 7.7μg RNA per blood tube and ranged between 2.5 to 14μg RNA per tube.

3.2.2.2 Taqman real time polymerase chain reaction (RT-PCR)
To compare expression of MxA relative to the house-keeping gene, GAPDH, quantitative RT-PCR was performed using the qScript™ One-Step Fast RT-qPCR Kit (95080-500, Quanta Biosciences). Primers and probes were custom designed and purchased from TIB MOLBIOL, New Jersey. The sequence of the forward and reverse primers were 5’-AgCCTCAAgATCATCAgCAATg and 5’-CACgATACCAAAgTTgTCATggA for GAPDH, and 5’-CAgCACCTgATggCCTATCA and 5’-TggAgCATgAAgAACTggATgA for MxA. Transcripts were detected by Taqman® probes for DNA detection, which was 5’ labelled with a fluorescent dye and 3’ labelled with a quencher. The sequence of the GAPDH probe was 5’-6JOECAgCAACCTgTTAgCAAC--TMR; the sequence of the MxA probe was 5’-6FAMAggCCAgCAAgCgCATCTCCA--TMR. Each 20μl reaction mixture contained manufacturer’s recommended concentrations of recombinant Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, dNTP, magnesium chloride, AccuStart Taq DNA polymerase, ROX Reference
Dye and stabilizers in addition to 200mM forward and reverse primer, 100nM probe, as well as 4uL RNA sample. The two different gene targets were amplified in separate wells, both in duplicate. A serial dilution of *in vitro* transcribed MxA RNA over a 10⁶-fold range of copy number was included in each assay to assess assay reproducibility. The reaction was processed in the ABI 7300 Real-Time PCR System. Cycling conditions consisted of cDNA synthesis at 50°C for 5 minutes and cDNA denaturation at 95°C for 30s, followed by two-temperature PCR cycling for 40 cycles at 95°C for 3s, then at 60°C for 27s for data collection. All samples obtained from one subject were processed in a single PCR run.

### 3.2.2.3 Data analysis for RT-PCR

The threshold cycle (CT) for MxA and GAPDH was determined by the ABI Prism 7300 Sequence Detection Software (PE Applied Biosystem, Foster City, CA, USA) as the mean plus 10 standard deviations of the first 15 cycles. The threshold cycle represents the number of PCR cycles required for the detection of fluorescence from the dye once the Taqman probe has been cleaved. For each RNA sample, threshold cycle was assigned for both MxA and GAPDH, then ΔCT was calculated as CT(MxA) – CT(GAPDH). This was normalized to a normalization standard sample of RNA, which is run every assay and was assigned the normalization ratio (NR) of 1. Finally, the normalization ratio of each sample was calculated as 2⁻ΔΔCT, where ΔΔCT is the difference between ΔCT’s of the test sample and the normalization sample. The normalization sample consists of RNA pooled from 15 healthy controls.

### 3.2.3 Neutralizing antibodies

Neutralizing Antibodies (NAbs) were measured by a luciferase reporter gene assay. Briefly, HL116 cells were seeded at 40,000 cells/well in a 96-well plate and incubated over night at 37°C. Patient sera were heat inactivated for 30 minutes at 56°C and a series of dilutions of the test serum, beginning at 1:10, was pre-incubated with an equal volume of 20U/ml of IFN-beta for 1 hour at 37°C. This gives a final dilution of 1:20 diluted serum and 10U/ml IFN-beta. We use a homologous system where the challenge antigen is the treatment interferon, i.e., samples from IFN-beta-1a treated patients were assayed on IFN-beta-1a and samples from IFN-beta-1b treated patients were assayed on IFN-beta-1b. Samples were added to the cells and incubated for 5 hours at 37°C. A dilution series of interferon was included with each plate run. Plates were allowed to cool for 10 minutes at room temperature, SteadyLite™ substrate was added and luminescence was read. Antibody titres were calculated according to the Kawade-Grossberg and results were given in tenfold reduction units (TRU)/ml defined as the dilution of antibody
required to reduce 10LU/ml of interferon activity to 1LU/ml. Samples greater than 20 TRU/ml were considered positive (Lam, et al., 2008).

3.2.4 Statistical analysis
The Kruskal-Wallis test with Dunn’s multiple comparison post test was used to compare NAb expression between healthy controls and the dynamics of MxA mRNA. Results did not follow a normal distribution therefore expressed as median values and were analysed using non-parametric tests. Differences in MxA expression between NAb positive and NAb negative groups were tested with the Mann-Whitney test. The correlation between NAb titres and MxA mRNA expression was determined with the Spearman’s rank test.

3.3 Results

3.3.1 MxA mRNA RT-PCR assay reproducibility
Inter-assay variability of the RT-PCR assay for MxA mRNA was evaluated by assaying fifteen RNA samples as in-house controls. These samples were selected from all the samples obtained for this study to represent a range of MxA mRNA expression (NR=0.8-60).

Inter-assay variability was evaluated by repeating the RNA samples on three separate days. The coefficient of variation for the inter-assay variability was 4% to 21% with an average of 12%. For similar assays, others have reported a 17-27% inter-assay variability and a 13% inter-assay variability (Capra, et al., 2007; Sellebjerg, et al., 2009). The high coefficient of variation can be attributed to the exponential nature of the Normalization Ratio.

3.3.2 MxA mRNA levels in healthy controls not receiving interferon-beta
MxA mRNA normalization ratios for healthy controls (HC) not receiving IFN-beta ranged from 0.29 to 4.44 with a median value of 0.96 ± 0.20 (95% confidence interval).

3.3.3 Effect of drug washout on MxA mRNA expression in one NAb negative MS patient treated with interferon-beta
To determine the effect of drug washout on MxA mRNA expression, we studied MxA expression in one NAb negative patient (treated with Rebif® 44μg SC) at pre and 2, 4, 6h post IFN injection subsequent to a 48h drug washout and compared them to MxA expression at pre and 2, 4, 6h post IFN injection subsequent to a 7-day drug washout. At pre IFN-injection, baseline MxA mRNA expression was lower with a 7-day drug washout compared to a 48h drug washout. However, MxA expression at 4 or 6 hours post IFN-injection did not appear different between
48h drug washout and 7 day drug washout (Figure 3.1). We concluded that a 7-day drug washout was not necessary for MxA expression measurements.

3.3.4 Dynamics of MxA mRNA expression in NAb negative MS patients treated with interferon-beta

Nine NAb-negative patients participated in the study. Median baseline MxA expression in NAb-negative (NAb-) patients (n=9) was significantly higher than that of non-injected healthy controls (HC, n=15), p<0.001. MxA mRNA expression was significantly different from the non-injected controls at 4, 6, 8, 10,12h post IFN injection (p<0.001; p<0.001; p<0.001; p<0.01; p<0.01, respectively) (Figure 3.2). Peak MxA mRNA expression was observed at 8h post IFN-injection. Six hours post injection was chosen for the subsequent study for MxA mRNA in NAb positive patients.

3.3.5 MxA mRNA in NAb positive MS patients treated with interferon-beta

Five patients had positive NAbs. We compared MxA mRNA expression of NAb positive (NAb > 20 TRU/ml) versus NAb negative patients at 6h post IFN injection. Relative MxA expression was lower in NAb positive patients compared to NAb negative patients (p=0.004, Mann-Whitney Test) (Figure 3.3). NAb titres did not correlate with either MxA expression at 6h post IFN (Spearman r=0.70, p=0.23) or with MxA induction (Spearman r=0.80, p=0.13) (data tabulated in Table 3.2). This could be due to the size of our sample, or because at these levels of NAb, MxA induction was negligible and “the grey zone” of NAb titres is lower than the NAb titres of the subjects included in the study.

3.3.6 A case report: effect of switching to a different interferon-beta product in a NAb positive patient

NAbs are cross reactive between the different IFN-beta preparations (Khan, and Dhib-Jalbut, 1998; Millonig, et al., 2009) and when switching therapy for NAb positive patients, it is generally not recommended to switch to another IFN-beta product (Polman, et al., 2010). However, in one report, a patient did not show an MxA response while on IFN-beta-1a therapy but showed an MxA response when receiving IFN-beta-1b (Gibbs, et al., 2006).

We investigated whether switching to another IFN-beta product would restore lost IFN-beta biological activity in one patient with low / moderate NAb titre (340TRU/ml, IFN-beta-1a as antigen; 194 TRU/ml, IFN-bea-1b as antigen). Blood samples were collected before, 2, 4, and 6 h after a SC IFN-beta-1a (Rebif® 44) administration and a SC IFN-beta-1b (Betaseron®)
administration. We measured MxA mRNA expression at each time point after IFN administration. Area under the curve (AUC) was calculated to compare overall changes after the administration of either drug. The AUC value for MxA mRNA after Betaseron® application was 13.0 and AUC after Rebif® application was 8.5. The AUC value of MxA mRNA was higher following Betaseron® administration as compared to Rebif® 44. Betaseron® application increased the biological effect of IFN compared to Rebif®. Median values of MxA mRNA following the two different IFN applications are tabulated in Table 3.3. The results indicate that administration of IFNbeta-1b may restore some bioavailability in patients with low NAb titres. Nonetheless, the increase of MxA mRNA did not reach levels observed in patients without NAb.

One 250μg dose of IFN-beta-1b (Betaseron®) contains 8 million international units (MIU) of activity and one 44μg dose of IFN-beta-1a (Rebif® 44) contains 12 MIU of activity. However, there is more protein weight in one dose of Betaseron® compared to Rebif® 44. A possible explanation for the restored bioactivity upon Betaseron® administration would be that more IFN-beta molecules were present to overcome the effects of NAb when Betaseron® was administered compared to when Rebif® was administered.

3.4 Discussion

Measuring MxA mRNA by real-time PCR can be used to monitor antibody mediated decreased IFN-beta bioactivity in treated MS patients. RT-PCR is reproducible and accessible in many laboratories. It is easy to perform and requires less time than currently available methods for measuring NAbs.

In this study, we investigated the significance of including a drug washout prior to blood draws for measurement of MxA. There was little difference between including a 48h drug washout and including a 7-day drug washout prior to measuring MxA mRNA expression, suggesting that a 7-day drug washout is not necessary for IFN-bioactivity measurements. We demonstrate that peak MxA mRNA expression occurs at 8h after an IFN-beta injection in NAb negative patients and the biomarker can be optimally measured at 4-12 hours. In NAb positive patients, MxA mRNA was attenuated at titres as low as 191 TRU/ml. Although our study sample was small, this is consistent with previous studies (Sominanda, et al., 2008).

We show in a case report that in one IFN-beta-1a treated patient with low NAb titre, switching to IFN-beta-1b may restore the biological activity of IFN-beta as measured by MxA mRNA. Although IFN-beta biological was not fully restored, MxA mRNA expression was higher after
IFN-beta-1b administration than with IFN-beta-1a administration. We speculate that this is due to the higher number of IFN-beta molecules in one dose of IFN-beta-1b compared to IFN-beta-1a.

Compared to NAb testing, bioavailability testing is a more direct way of measuring drug efficiency. If IFN-beta does not reach its receptor, all of its beneficial effects can be assumed lost regardless of antibody levels. Although NAb measurements can predict the likeliness of antibody persistence (Sorensen, et al., 2005; Sominanada, et al., 2009), it would be in the interest of a NAb positive patient to switch to an alternative therapy rather than to wait for antibodies to disappear as novel therapies are now available for the treatment and management of multiple sclerosis. Nonetheless, IFN-beta remains the most common therapy for MS and NAbs remain as an important issue for MS patients as long-term safety profiles of the newer MS drugs have yet to be established.

In conclusion, in vivo MxA mRNA levels can be optimally measured at any time between 4-12h after an IFN-beta injection. MxA bioavailability was lower in NAb negative compared to NAb positive patients. MxA bioavailability testing helps determine how much NAbs affect the biological activity of IFN in NAb positive individuals.

3.5 Acknowledgements

We would like to thank individuals who participated and donated blood for the study. We are grateful for Tariq Aziz and staff at the UBC MS Clinic for guidance and assistance in phlebotomy.
Figure 3.1 MxA Expression pre and post IFN-injection in one NAb negative patient with 48h drug washout (open circles, ◦) or with 7 day drug washout (filled circles, ●). Baseline MxA expression is higher with 48 hours drug washout compared to a 7-day drug washout. However, MxA expression at 4 or 6 hours post IFN-injection was not significantly different between 48h drug washout and 7 day drug washout.
Figure 3.2. MxA mRNA Expression in non-injected healthy controls (□, open squares) and in NAb Negative IFN-beta treated patients at various time points after IFN-beta injection (●, filled circles, SC IFN-beta-1b; ○, shaded circles, SC IFN-beta-1a; ○, open circles, IM IFN-beta-1a).

**p<0.001; *p<0.01, Dunns Test for Multiple Comparisons**
Figure 3.3. MxA expression in NAb Negative and NAb Positive IFN-beta treated MS Patients, 6 hours post IFN-injection. Median values are shown for each NAb category. Relative MxA expression was lower in NAb positive patients compared to NAb negative patients (p=0.0016).
Table 3.1.

Effect of time after IFN-beta injection on MxA gene expression.

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<th>Reference</th>
<th>Times Examined (h)</th>
<th>Conclusion</th>
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<td>3, 9, 12, 24, 48</td>
<td>Strongest responses at both 9 and 12h, response waned but not at baseline by 48h</td>
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<td>Gilli et. al., 2005</td>
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<td>Increase at 3h, peaked at 12h, declined after 24h</td>
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</table>
Table 3.2

Individual NAb titres, MxA expression and MxA induction of five NAb positive patients. NAb titres given in TRU/ml; MxA expression given as normalization ratio (NR); MxA induction given as ratio of MxA expression post IFN divided by MxA expression at baseline (pre-IFN).

<table>
<thead>
<tr>
<th>NAb Positive Patient #</th>
<th>Interferon Treatment</th>
<th>NAb (IFN-beta-1a antigen) (TRU/ml)</th>
<th>NAb (IFN-beta-1b antigen) (TRU/ml)</th>
<th>MxA Expression at baseline (pre-IFN) (NR)</th>
<th>MxA Expression at 6h post IFN (NR)</th>
<th>MxA Induction (MxA Expression post IFN / MxA Expression pre-IFN)</th>
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<tr>
<td>1</td>
<td>Betaseron®</td>
<td>793</td>
<td>734</td>
<td>2.4</td>
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<td>2</td>
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<td>314</td>
<td>191</td>
<td>0.5</td>
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<td>3</td>
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<td>4</td>
<td>Rebif® 44</td>
<td>345</td>
<td>204</td>
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<tr>
<td>5</td>
<td>Avonex®</td>
<td>2575</td>
<td>498</td>
<td>1.3</td>
<td>1.5</td>
<td>1.2</td>
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Table 3.3.
Median values of MxA mRNA following the two different IFN applications. No significant difference between different time points could be observed between the two different interferon products.

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<td>Baseline</td>
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<td>Post 2h</td>
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<td>Post 4h</td>
<td>4.6</td>
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<tr>
<td>Post 6h</td>
<td>6.8</td>
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3.6 References


General discussion
4.1 Summary

In Chapter 2, we describe the optimization of a reporter gene assay to measure neutralizing antibodies to interferon-beta. We explored several parameters to optimize the assay including cell density, plate colour, plate incubation time, the amount of added substrate, as well as different methods to interpret raw data. The optimized method is sensitive and specific, easy to perform, less arduous, and requires less time than existing methods to measure NAbs. The diagnostic test has been approved by the Medical Services Commission in British Columbia and has been made available to all prescribing neurologists in Canada through the inter-provincial agreements.

In Chapter 3, we looked at one important parameter in standardizing MxA mRNA measurements in assessing the biological response of IFN-beta: what is the optimal time to draw blood after an IFN-beta injection. We reinstated the Real-Time PCR assay to measure MxA mRNA and assessed its reproducibility. The reproducibility of the assay was similar to other assays used to measure MxA mRNA. In IFN-injected MS patients without NAb, MxA mRNA expression was significantly higher than healthy controls at 4, 6, 8, 10 and 12 hours after IFN administration. Peak MxA mRNA expression was observed at 8h after an IFN-beta injection. This is consistent with earlier studies that MxA mRNA can be measured as early as 4h. We found that in NAb positive patients, the biological response was attenuated at titres as low as 191 TRU/ml. In our sample, MxA mRNA expression did not correlate with NAb titre as we hypothesized. This may be due to our small sample size, or may be attributed to inclusion of patients with NAb titres higher than “the grey zone” of low to moderate levels of antibodies and thus had negligible MxA mRNA expression.

NAb may help explain IFN-beta treatment failure and the measurement of MxA mRNA is a valuable tool for monitoring antibody mediated decreased bioactivity in treated MS patients, but it is prudent to keep in mind that it is not a surrogate marker for the effect of IFN-beta on MS the disease. IFN-beta is effective in reducing relapse rates in only about 30% of treated MS patients (The IFN-beta Multiple Sclerosis Study Group and the UBC MS/MRI Analysis Group, 1995; Jacobs, et al., 1996; PRISMS Study Group, 1998). The integration of clinical information and other paraclinical tools such as MRI will continue to guide the neurologist in evaluating the effectiveness of IFN-beta therapy, and adapting treatment strategies against multiple sclerosis.
Given the long-term experience and good safety profile of interferon-beta, the DMD will remain a mainstay in the treatment of MS despite the emergence of newer drugs. However, immunogenicity to the drug and anti-interferon-beta antibodies will continue to be an issue.

**4.2 Future challenges**

**4.2.1 Modifications to interferon-beta to reduce immunogenicity**

To reduce immunogenicity and to improve drug solubility, PEGylation is becoming a popular modification amongst therapeutic proteins. The process covalently conjugates biological molecules with polyethylene glycol (PEG), a non-toxic, non-immunogenic polymer, and changes the physical and chemical properties of the molecule, improving pharmacokinetic behaviour of the drug (Veronese and Mero, 2008). PEGylated IFN-beta-1b was shown to have maintained its functional activities while the formation of aggregates of the highly hydrophobic, aggregation-prone protein was ameliorated. The improvement in drug solubility decreased immunogenicity of the drug and the PEGylated molecule had an expanded AUC exposure compared to the unmodified protein (Basu, et al., 2006). PEGylated IFN-beta-1a (PEG Intron®), was shown to have a longer half-life when compared with the native protein. The drug has been shown to be safe and effective in reducing hepatitis C viral load and maintaining sustained viral suppression in patients when combined with ribavirin (Youngster, et al., 2002). Currently, a phase III trial is underway to evaluate the efficacy and safety of PEGylated interferon-beta-1a (BIIB017) in subjects with relapsing MS (US FDA, 2010).

In addition to PEG, dodecylmaltoside, a nontoxic alkylsaccharide surfactant, has also been shown to reduce aggregation of interferon-beta *in vitro* and to reduce its immunogenicity *in vivo* (Rifkin, et al., 2010).

**4.2.2 Predictive markers for interferon-beta response**

Interferon-beta is partially effective in multiple sclerosis and non-response can partially be explained by the presence of anti-IFN-beta antibodies. Breakthrough disease can occur in patients receiving therapy who show no sign of impaired biological activity (Hesse, et al., 2010). The underlying explanation of this variability may be attributed to the highly heterogeneous disease.

A recent theme in the field has been the search for predictive markers for IFN-beta response. T-bet, which is a transcription factor which directs Th1 lineage commitment (Szabo, et al., 2000), has been suggested as a predictor of IFN-beta response (Drulovic, et al., 2009). This is
supported by a recent report from Axtell and colleagues that IFN-beta was effective in reducing EAE symptoms induced by Th1 cells but exacerbated disease induced by Th17 cells. In RRMS patients, IFN-beta non-responders had higher IL-17F concentrations compared to responders, suggesting that IFN-beta is effective in the Th1 type of MS, but not in the Th17 type of MS (Axtell, et al., 2010).

On the other hand, IFN-beta has been reported to inhibit human Th17 cell differentiation as a mechanism of action of the drug (Ramgolam, et al., 2009). Durelli and colleagues showed that the percentage of Th17 cells was increased during active MS but no change was observed in Th1 cells and IFN-beta-induced apoptosis was significantly greater in Th17 cells compared to Th1 cells. As greater IFNAR1 (interferon alpha receptor 1) was expressed by Th17 cells, the authors suggested that Th17 cells might make them a selective target for IFN-beta therapy (Durelli, et al., 2009). In vitro studies have also shown that IFN-beta can inhibit cytokines which induce Th17 cell differentiation, as well as induce cytokines which suppress Th17 cell differentiation. Moreover, IFN-beta was shown to directly suppress naïve T-cells’ differentiation into Th17 cells by inhibiting Th17 cell lineage markers (Zhang and Markovic-Plese, 2010).

Genome-wide scans are another approach to identify surrogate markers that correlate with IFN-beta responsiveness in MS. One study identified allelic variants in the glutamatergic system which influence response to IFN-beta therapy (Comabella, et al., 2009). Another study identified differentially expressed genes in the longitudinal transcriptional profile of IFN-beta treated patients within two years of treatment initiation to identify time dependent interactions between IFN-beta regulated genes (Goertsches, et al., 2010).

One future challenge will be the continued efforts in dissecting the mechanisms of IFN-beta, which will allow to better understand multiple sclerosis and to identify surrogate markers which reliably correlate with responsiveness to IFN-beta therapy in MS.
4.3 References


Appendix

Ethics Certificates
**ETHICS CERTIFICATE OF FULL BOARD APPROVAL**

<table>
<thead>
<tr>
<th>PRINCIPAL INVESTIGATOR:</th>
<th>INSTITUTION / DEPARTMENT:</th>
<th>UBC CREB NUMBER:</th>
</tr>
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<tbody>
<tr>
<td>Joel J.F. Oger</td>
<td>UBC/ Medicine, Faculty of/Medicine, Department of</td>
<td>H08-01535</td>
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**INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:**

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**CO-INVESTIGATOR(S):**

- Lorne F. Kastrukoff
- Virginia A. Devonshire
- Stanley A. Hashimoto
- Ebrima Gibbs
- Anthony Traboulsee
- Regina Lam
- Peter R. Rieckmann
- John P. Hooge

**SPONSORING AGENCIES:**

- Donations for Health Science Research - "Myasthenia Gravis Research"
- Dr. Schlegel Healthworld AG - "Setting-up, validating and evaluating the clinical usefulness of laboratory tests to measure antibodies to interferon in multiple-sclerosis patients"
- Various Pharmaceutical Companies - "Setting-up, Validating and Evaluating the Clinical Usefulness of Laboratory Tests to Measure Antibodies to Interferon in Multiple Sclerosis Patients"

**PROJECT TITLE:**

Evaluating the bioavailability of interferons in treated multiple sclerosis patients

**THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES:** August 5, 2009

The full UBC Clinical Research Ethics Board has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

**REB FULL BOARD MEETING REVIEW DATE:**

August 5, 2008

**DOCUMENTS INCLUDED IN THIS APPROVAL:**

<table>
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<th>Document Name</th>
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</table>
CERTIFICATION:

In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

Approval of the Clinical Research Ethics Board by one of:

Dr. Gail Bellward,
Chair
ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR: Joel J.F. Oger
DEPARTMENT: UBC/Medicine, Faculty of Medicine, Department of
UBC CREB NUMBER: H08-01535

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Evaluating the bioavailability of interferons in treated multiple sclerosis patients

THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: July 30, 2010

The UBC Clinical Research Ethics Board Chair or Associate Chair, has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

DOCUMENTS INCLUDED IN THIS APPROVAL:
healthy consent 28Jul09 annual rev.
ethics - subject consent 28Jul09 annual rev.

APPROVAL DATE: July 30, 2009

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2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
The UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

Approval of the Clinical Research Ethics Board by one of:

Dr. Peter Loewen, Chair
Dr. James McCormack, Associate Chair
ETHICS CERTIFICATE OF EXPEDITED APPROVAL:
AMENDMENT

PRINCIPAL INVESTIGATOR: Joel J.F. Oger
DEPARTMENT: UBC/Medicine, Faculty of Medicine, Department of
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REMINDER: The current UBC CREB approval for this study expires: July 30, 2010

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The amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

Approval of the Clinical Research Ethics Board by one of:

Dr. Peter Loewen, Chair
Dr. James McCormack, Associate Chair
**ETHICS CERTIFICATE OF EXPEDITED APPROVAL:**

**AMENDMENT**

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PROJECT TITLE:
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EXPIRY DATE OF THIS APPROVAL: June 28, 2011

APPROVAL DATE: June 28, 2010

CERTIFICATION:
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Dr. James McCormack, Associate Chair