INTERFERON-BETA TREATMENT IN MULTIPLE SCLEROSIS: ANALYSIS OF NEUTRALIZING ANTIBODIES

Ajith Sominanda
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Stockholm 2008
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To My parents,
Dammi, Sanduni & Nethmi
ABSTRACT

Multiple sclerosis (MS) is a disabling chronic neurological disease with a significant impact on patients’ lives. There is no cure for MS, but recombinant interferon beta (IFN-β) is currently the most established disease modifying therapy. One of the major clinical problems of IFN-β is the development of neutralizing antibodies (NAbs) that interfere with the clinical efficacy of the drug. Still, in the medical community, there is some skepticism regarding the clinical use of NAb testing and its interpretation at the individual level. The overall aim of this thesis was to provide evidence-based knowledge which will be useful in clinical management. To some extent, such information could help in understanding similar scenarios for the many emerging biopharmaceuticals that are potentially immunogenic and might induce antibodies which could interfere with clinical efficacy.

Biological study materials were blood samples from IFN-β treated MS patients that were referred from neurology units in Sweden, Norway and Finland to the specialized NAb laboratory in the Neurology Division at the Karolinska University Hospital in Huddinge. NAbs were measured using the MxA induction assay and in vivo IFN-β bioactivity was measured by the in vivo MxA mRNA assay.

We observed an overall 32% of NAb positivity in MS patients who were tested for NAbs from 2003 to 2006. Weekly low-protein-dose intramuscular IFN-β was found to be the least seroprevalent and immunogenic product compared to the weekly-high-protein-dose subcutaneous IFN-β preparations. A titer around 150 TRU/ml marked an important IFN-β bioactivity level for the NAb titers as a significant in vivo drug response was retained up to this level. Titors above 600 TRU/ml were associated with complete loss of IFN-β bioactivity. Fluctuation of NAb titers was assessed across the functionally critical titer of 150 TRU/ml and showed that the majority (72%) of patients were stable, especially the two extreme ends of the titer spectrum i.e. negative (less than 10 TRU/ml) and very high titers (>600 TRU/ml). As expected, titers close to 150 TRU/ml appeared more prone to fluctuation across this level, i.e. offering a greater chance of regaining good IFN-β bioactivity, or, reversely, a greater risk of losing bioactivity. These observations were not influenced by treatment duration or sampling interval between consecutive NAb tests. We failed to observe an increased NAb positivity when referrals indicated an impression of disease worsening.

Conclusion: Here, we studied several clinically relevant aspects of IFN-β NAbs. We identified differences between the IFN-β preparations regarding the frequency of NAb induction, which we refer to as seroprevalence, but also regarding titer levels of induced NAbs, which we refer to as immunogenicity. In a population-based sample of MS patients we demonstrated that these parameters vary between IFN-β preparations. Further, we identified titer levels that are critical for retaining the in vivo bioactivity of IFN-β, vital for the interpretation of individual NAb tests. We showed that NAb titers are commonly stable with a greater tendency of fluctuation of mid-range titers. Finally, we showed that a clinical impression of worsening is poorly predictive of NAb status, implicating the necessity of regular NAb testing. Although NAb testing and its interpretation remains a complex area, our results have provided clinically useful knowledge of relevance in the management of MS patients treated with IFN-β.
LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Ajith Sominanda, Uros Rot, Mari Suoniemi, Florian Deisenhammer, Jan Hillert and Anna Fogdell-Hahn
   Interferon beta preparations for the treatment of multiple sclerosis patients differ in neutralizing antibody seroprevalence and immunogenicity
   Multiple Sclerosis 2007; 13:208-214

II. Ajith Sominanda, Jan Hillert, Anna Fogdell-Hahn
    In vivo bioactivity of interferon beta in multiple sclerosis patients with neutralizing antibodies is titer dependent.

III. Ajith Sominanda, Jan Hillert, Anna Fogdell-Hahn
     Neutralizing antibodies against interferon beta: fluctuation is modest and titer dependent
     European Journal of Neurology (in press)

IV. Uros Rot, Ajith Sominanda, Anna Fogdell-Hahn, Jan Hillert
    Impression of clinical worsening fails to predict interferon beta neutralizing antibody status
    Journal of International Medical Research (in press)

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<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen-4</td>
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<tr>
<td>EAE</td>
<td>Experimental allergic encephalomyelitis</td>
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<td>EBV</td>
<td>Epstein-Bar virus</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>HHV-6</td>
<td>Human Herpesvirus-6</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>Interferon-alpha</td>
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<td>IFNAR</td>
<td>Interferon receptor</td>
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<td>IL7Rα</td>
<td>Interleukin-7 receptor-alpha</td>
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<td>JAK1</td>
<td>Janus kinase</td>
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<tr>
<td>LU</td>
<td>Laboratory units</td>
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<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MxA</td>
<td>Myxovirus resistant protein A</td>
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<tr>
<td>NAbs</td>
<td>Neutralizing antibodies</td>
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<tr>
<td>PML</td>
<td>progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary progressive multiple sclerosis</td>
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<tr>
<td>rIFN-β</td>
<td>Recombinant interferon-beta</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transduction and activators of transcription</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitors of metalloproteinase-1</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRU/ml</td>
<td>Ten-fold reduction units/ml</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cellular adhesion molecule</td>
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1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic neurological disease of the central nervous system (CNS), which is characterized by multiple areas of demyelination and scarring (sclerosis) in brain and spinal cord. The disease usually starts in young adulthood and is more common in females. However, it does not shorten the lifespan greatly but the majority of the patients gradually accumulate neurological dysfunction and disability as the disease progresses [1].

1.1.1 Clinical features, course of the disease and diagnosis

MS is heterogeneous in clinical presentation and also in subsequent course of the disease. Clinical features of MS results from lesions that interrupt conduction of nerve signals in CNS whereas the peripheral nerves are spared [2]. Three main clinical courses of MS can be identified: Relapsing remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS). Eighty-five percent of all MS patients initially have RRMS, which is characterized by episodes of neurological symptoms and signs (relapses) followed by periods of recovery (remissions). These relapses and remissions can follow a variable course and severity. Initial relapses usually recover fully, but later, the recovery is partial leaving residual neurological deficits. After about 10-20 years, the disease commonly switches into a phase of slow progressive neurological deterioration with less relapsing remitting character, the secondary progressive MS (SPMS) [3]. Ten to 15 percent of all MS patients experience steady neurological disability progression without preceding relapses, which is known as primary progressive MS (PPMS) [4]. Intermediate clinical types also exist: malignant form which is rapidly progressive and often lethal and a form of mild course of RRMS with minimal disability that allows normal life even long term (benign MS).

During relapses, patients experience functional impairment and as the disease progresses, they accumulate disability due to irreversible tissue damage. On average, within 10 years of onset of symptoms, patient’s household and employment work will be affected; within 15-20 years patients are unable to walk unaided; within 25-30 years, they may be unable to walk at all [3, 5].

Figure 1: Natural history of MS
Clinical features of MS involve a wide spectrum of neurological symptoms and signs: motor, sensory, autonomic and higher functions of the nervous system. The most frequent presenting symptom is a sensory disturbance, often with paraesthesiae, followed by other neurological problems in order of frequency [1]:

1. Paraesthesiae
2. Limb weakness
3. Optic neuritis
4. Diplopia
5. Vertigo
6. Disturbance of micturition
7. Others neurological symptoms and signs

Although there is no pathognomonic clinical feature or specific diagnostic test for MS, the diagnosis is most often readily made based on a typical clinical picture in combination with typical findings on magnetic resonance imaging (MRI) and laboratory investigations i.e. cerebrospinal fluid (CSF) analysis. However, other demyelinating diseases that can mimic the clinical presentation of MS need to be excluded. The currently used McDonald’s criteria for an MS diagnosis include clinical as well as paraclinical findings, i.e. MRI and CSF analysis [6].

There have been several efforts in setting up and revising diagnostic criteria for MS [7-11]. Since early nineteen eighties, Poser criteria were used and gained wide acceptance in both clinical and research settings [12]. But they retained some weaknesses and not all patients neatly fit into the diagnosis such as progressive form of the disease. In 2001, McDonald’s criteria were introduced, which incorporated MRI along with the clinical evidence to demonstrate dissemination of lesions in space and time. Laboratory investigations such as CSF analysis have also been included to expand the spectrum of diagnosis. These revised criteria can address a variety of presentations such as "monosymptomatic" disease suggestive of MS, typical relapsing-remitting disease, insidious progressive form of disease without evidence of relapses and remissions and hence being supportive for clinician with a more secure diagnosis of MS even at an early stage of the disease [6], which is critical for starting an early treatment in MS.

1.1.2 Etiology and pathogenesis

MS has been known and documented for more than a century. Jean-Martin Charcot (1825-1893) provided the first systematic clinical and neuropathologic account of the disease, describing the demyelinating process that leads to formation of MS plaque with a glial scar. Even though extensive progress has been made, the etiology of MS still remains elusive [1]. Currently, the evidence suggests that MS is a disease with complex genetic trait and the cause of the disease may be a combined interplay between genes and environment.

1.1.2.1 Genetics and environment

Ethnic populations vary in disease prevalence and the highest rates are reported among the people of north and west European origin. Twin studies show a higher concordance rate (25-30%) among monozygotic twins compared to dizygotic twins (3-5%) and to the normal population (0.1-0.4%). Nineteen percent of MS patients have one or more affected family members suggesting familial clustering of the disease [13-15]. Genome wide screening has shown that one or more human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC) region of
chromosome 6, are linked to MS susceptibility [16, 17]. Of these, HLA-DR and HLA-DQ genes seem to confer the highest risk of disease susceptibility [Reviewed in 18, 19], while HLA-A0201 has been shown to be protective in MS [20]. The HLA region is known for the presence of genes that are important for functions in the immune system, and thereby, the HLA associations in MS can be regarded as evidence for the autoimmune hypothesis in MS pathogenesis. A number of non-HLA genes have been reported to be associated with disease susceptibility: Cytotoxic T-lymphocyte antigen-4 (CTLA-4) [21], interferon-gamma (IFN-γ) [22], Apolipoprotein E (APOE) [23] although none of these have been convincingly confirmed. However, recently the interleukin-7 receptor-alpha (IL7R) gene has been unequivocally identified as the first non-HLA risk gene in MS [24].

Genetic factors per se do not fully explain the etiology of MS. Non-genetic or environmental contributions to the etiology have been implicated as triggers of the disease in genetically susceptible individuals. There is geographical variation in disease prevalence with arctic and temperate regions showing high-risk and a low prevalence towards equator. This could be attributed to both genes and environment [25, 26]. Infections as environmental triggers in MS have been implicated in numerous reports; occurrence of MS relapses after viral infections [27] and ‘MS epidemics’ in isolated communities like the Faroe Islands [28]. These observations led to the speculation of infective etiology in MS [Reviewed in 29]. But failure to isolate infective agents in MS patients or lesions in a consistent manner has made it impossible to directly correlate infections as a causative factor.

1.1.2.2 The disease ‘trigger’ and autoimmunity

Human and microbial sequencing studies have revealed the existence of similar molecules between some microbial-pathogens and humans. When such microorganisms invade human body mounting an immune response against the infection, there is a possibility for ‘self’ proteins to be mistakenly attacked; the process is termed molecular mimicry. Human herpes virus (HHV-6) [30] and Epstein-Barr virus (EBV) [31] are two examples of viral agents with gene sequences that code for proteins that mimic components of myelin. These are very successful human pathogens that infect more than 80% of the population and maintain latent infections throughout life. HHV-6 is both neurotrophic and lymphotropic while EBV is mainly lymphotropic [Reviewed in 29, 32], and thus capable of residing in the appropriate tissue compartments that are pathologically relevant in MS. Alternatively to molecular mimicry, it has been proposed that during viral replication, some host proteins in the brain are incorporated into the viral particles and subsequently the incorporated host proteins are targeted by the host immune system. What is attractive with this hypothesis compared to molecular mimicry, which is often restricted to a single peptide cross presentation, is the presentation of whole sets of proteins from target tissue [33].

Thus, given the genetic predisposition and a favorable environmental condition, the autoreactive immune cells (T and B lymphocytes) get activated in the periphery, perhaps by molecular mimicry, and start to express surface adhesion molecules. These cells adhere to endothelium of cerebral blood vessels, cross the blood brain barrier (BBB) by diapedesis and set up the initial wave of inflammation by disrupting BBB. In the CNS, these cells encounter the candidate myelin antigens presented by resident or migrated antigen presenting cells. Re-activated T cells start a cascade of immunological effector mechanisms: cytotoxic T-cell killing, antibody production, complement expression and resident microglial activation. These cells secrete inflammatory
cytokines and, along with activated cytotoxic T cells, antibodies and complements, start to attack myelin-oligodendrocyte units which trigger the process of demyelination [Reviewed in 34, 35].

Experimental evidence from the MS animal model experimental autoimmune encephalomyelitis (EAE) has supported T-cell mediated autoimmunity in MS. EAE is typically induced by injecting myelin proteins along with an adjuvant and then the disease can even be adoptively transferable to a healthy animal using activated myelin-specific T cells. This model replicates clinical and neuropathologic features found in MS. However, the key difference between MS and EAE is that for EAE the cause is known; but in MS it is still unknown [Reviewed in 36, 37].

1.1.2.3 Neuropathology

The characteristic neuro-pathologic feature in MS is the presence of large confluent plaques or primary demyelination with reactive glial scarring [38]. Most lesions are disseminated around small blood vessels of periventricular areas, corpus callosum, cerebellum, cervical cord and optic nerves. Immunohistochemical studies on MS lesions have demonstrated antibody deposition, up-regulation of histocompatibility antigens, cytokines, chemokines and adhesion molecules [39-41]. In addition, lesions are dominated by the presence of T lymphocytes and macrophages or activated microglia [42]. Presence of these cells and related molecules endorse the idea of immunological basis of the inflammatory demyelination process in MS [43]. However, the composition of these lesions is heterogeneous among different individual patients. Lucchinetti [40] and co-workers studied 51 biopsies and 32 autopsies that contained actively demyelinating lesions, which were analyzed by using a large number of immunological and neurobiological markers. Based on this analysis, they described four basic patterns of demyelination characterized by myelin protein loss, location and extension of plaques, patterns of oligodendrocyte destruction, and complement activation. All patterns of demyelination occurred in a background of T-cell and macrophage-dominated inflammation.

a.) **Pattern I:** the demyelination was observed to occur in the presence of T cells and activated macrophages or microglia alone. This suggested that demyelination was mainly mediated by cytotoxic T cells and by neurotoxic products released from macrophages or microglia.

b.) **Pattern II:** this was similar to the basic pattern observed in pattern I but was characterized by antibodies and complement mediated demyelination.

c.) **Pattern III:** selective loss of myelin-associated glycoproteins (MAG) in periaxonal myelin and distal oligodendrogiopathy were observed. The underlying mechanism was similar to hypoxia-like tissue injury that is observed in acute white matter stroke.

d.) **Pattern VI:** basic pattern was similar to pattern I but associated with extensive demyelination, oligodendrocyte death and tissue injury.

Overall, the pattern I showed a similarity to T-cell mediated autoimmune encephalomyelitis whereas pattern II showed a similarity to T cells plus antibodies mediated encephalomyelitis. The patterns III and IV were suggestive of a primary oligodendrocyte dystrophy similar to virus or toxic induced demyelination rather than autoimmunity [40].

Reactivity of astrocytes was generally observed in all patterns of demyelinating lesions suggestive of damage to BBB in MS [40]. Such damage opens up of CNS parenchymal tissues to vascular
compartment and favors more immune cell trafficking into CNS [44]. BBB is a critical site for the action of IFN-β, which seals off damaged BBB [45], and for natalizumab, which blocks activated immune cells trafficking across BBB [46]. These two drugs are potent in reducing inflammation and hence decreasing the disease activity by reducing relapse rate and severity. Therapeutic benefit from these agents thus provides an indirect evidence for inflammatory and immunological basis of disease pathogenesis.

In contrast to active MS lesions, chronic lesions also have some degree of ongoing inflammatory demyelination [47]. It is now evident that inflammation is not only localized to typical white matter regions, but also to the areas of normally appearing white matter [48] and to the gray matter of brain and spinal chord [49, 50].

The most critical pathological event may be the irreversible axonal damage. Inflammation can result in physiologic nerve conduction block which reflects as acute neurological dysfunction, which is often reversible during early relapses [51]. However, the bulk of axonal damage has been found to be occurring even during early lesion formation when there is active demyelination. Chronic axonal damage and progressive white matter loss, which overrides the rate of regeneration and remyelination, lead to brain atrophy and accumulation of permanent neurological disability [52-54].

Most of the current therapies are targeted towards the inflammatory phase of the disease [55]. Once the disease becomes chronic and progressive with less inflammation, these drugs appear to be failing. Currently, the need for more therapeutic attention in later phase of the disease i.e. neuro-protective and neuro-repairing drugs, has been emphasized [55].

In summary, MS pathology has two principle observations: inflammatory demyelination and nerve and glial degeneration in CNS, giving the idea that MS is a two–stage disease [32]. The net effect of the complex interplay of inflammation, demyelination, and axonal and glial degeneration may be reflected in the clinical picture, in the course of the disease and in the individual differences that have been observed during therapeutic response.

1.1.3 Treatment

Since MS is a chronic and largely a progressive disease, treatment is geared to address many aspects of MS patient’s life as a whole once the diagnosis is made. MS has major impacts on the life style, employment, recreational activities, family life, pregnancy and childcare, independence etc. Eighty percent of RRMS patients experience relapses during early stage of the disease, which prompts medical intervention. This is later followed by the need of rehabilitation and care giving as the disability accumulates [1].

Currently there is no cure for MS, but recent advancement in the understanding of the disease pathogenesis, improvement in clinical trial methods, widespread application of MRI and progress in the field of biotechnology has offered tremendous new opportunities for drug development, and thereby availability of better therapeutic options for MS patients. Currently, immunomodulatory treatment is the mainstay of therapeutics in MS and recombinant interferon beta (rIFN-β) and glatiramer acetate were the first such drugs which were shown to be effective in reducing relapse rates by 30-40%. These drugs still serve as the standard treatment in MS even though the clinical effectiveness is modest. Natalizumab is a newly emerged drug with superior
clinical efficacy over IFN-β and glatiramer acetate. However, it has encountered a safety issue with the emergence of progressive multifocal lekoencephalopathy (PML) during the clinical trials [56-58]. Now it is back in the market with close surveillance measures for the emergence of PML.

A basic therapeutic and rehabilitation management of MS can be outlined as follows [1]:

a.) Treatment of acute relapses.
b.) Symptomatic treatment and rehabilitation.
c.) Immuno-suppression drugs and disease modifying therapy.
d.) Therapeutic approaches for remyelination, repair and regeneration of nerve tissues.

1.2 INTERFERON-BETA TREATMENT IN MULTIPLE SCLEROSIS

1.2.1 Type I interferons

IFN-β is a cytokine in the type I interferon family with diverse functions in the human body. Of the complex biological functions, the effect on the immune system is considered to be therapeutically beneficial in MS, which is broadly known as an immunomodulatory effect.

1.2.1.1 History of interferons

In 1957, Isaacs and Lindenmann discovered a substance that conferred protection against viral infections in animal cells [59, 60], which they called interferon due to its ability to 'interfere' with propagation of viral infection. Subsequently, these substances were identified as a mixture of IFN-α and IFN-β and termed type I IFNs. In the 1970s, a similar protein with slightly lower antiviral activity was discovered and was termed ‘type II interferon’ or ‘immune interferon’ [61].

With the expansion of knowledge in the field and for the necessity of consistency in the classification system, an international panel agreed on a nomenclature system for interferons based on gene and amino acid sequence homology, and on physical and biological properties [62, 63]. Hence, interferons were designated as IFN-α, IFN-β, and IFN-γ etc. Type I IFNs included IFN-α and IFN-β, while the type II family included only one cytokine, the IFN-γ. Currently, the type I IFN family has been expanded with the discovery of more molecules and a type III family of interferons has also been introduced, which includes interferon lambda (IFN-λ) [64].

1.2.1.2 Biology of type I interferons

A number of different type I IFNs has been found in vertebrates. Of these, IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω have been identified in humans. Type I IFNs are induced in response to viruses, double stranded RNA (dsRNA) and growth factors. Any nucleated cell infected with virus or products of viral replication are capable of producing type I interferons [Reviewed in 65].

IFN-α and IFN-β are predominantly produced by precursors of dendritic cells [66] and fibroblasts respectively [67]. Genes clustered on short arm of chromosome 9 codes for type I IFNs. IFN-α has nearly 13 protein subtypes that are encoded by several genes, and each has unique functional profiles although they have considerable structural similarities. However, IFN-β or IFN-ω are single functional proteins coded by single genes [Reviewed in 68]. There is 30% amino acid sequence similarity between IFN-α and IFN-β and they have approximately the similar peptide length consisting of 165 to166 amino acids respectively.
X-ray crystallographic study on rIFN-β1a (Avonex) by Karpusas and co-worker reported that IFN-β is roughly a cylindrical structure consisting of five helices (labeled A to E in figure 2). The amino acid position at Asn-80 contains the site of glycosylation. In addition, Cys-31 of the AB loop and Cys-141 of the DE loop form a disulphide bridge, which is thought to be important in interferon receptor engagement. In the crystalline form, IFN-β tended to be dimerized at a Zinc containing site [69, 70].

**Figure 2:** Schematic representation of the crystallographic dimer of human IFN-β. The modeled portion of the carbohydrates and part of the zinc-binding site are also shown. The sphere corresponds to the zinc ion. Helices and N and C termini are labelled. The AB loop is colored green.

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All type I interferons bind to a similar multimeric receptor system, which consists of two subunits (IFNAR-1 and IFNAR-2), and are found on most of the cells. Receptor-ligand interaction activates classic JAK-STAT signaling cascade, eventually leading to expression of interferon inducible genes. But, several other signaling pathways are cross-linked to regulate the complex biological functions of IFNs such as antiviral, anti-proliferative, immunomodulatory and developmental functions (figure 3) [Reviewed in 65].

**Interferon signaling:**

a. The binding of IFN-α, IFN-β or other type I interferons results in dimerization of IFNAR1 and IFNAR2 subunits, and leads to autophosphorelation of receptor associated intracellular family of tyrosine kinases (TYK2) and Janus kinases (JAK1). Cook et al showed that IFNs utilize different domains of IFNARs for binding and thereby suggesting a different signaling specificities [71].

b. Phosphorylated JAKs then dimerize another set of transcription factors named signal transduction and activators of transcription STAT1 and STAT2. Different STAT proteins are activated and bind in different combinations in response to different type of IFNs bound to the receptor system, thus giving another level of functional specificity for different interferons in different cell types.
c. The heterodimeric STAT1-2 complex combine with intracellular interferon regulatory factor 9 (IRF9) to form transcriptional unit (or in other situations, the homodimeric or heterodimeric forms of STATs with different IRFs) called IFN-stimulated gene factor (ISGF3) complex, which then translocate into the nucleus and binds a sequence within promoter regions of IFN-stimulated genes (ISGs), the IFN-stimulated response element (ISRE). The corresponding promoter sequences for IFN-γ-induced gene expression is known as the IFN-γ-activated sites (GAS) elements. Hundreds of ISGs have been identified in the genome, having ISREs or GAS elements or combinations.

Thus, products of type I ISGs mediate a number of biological activities. ‘Antiviral state’ is induced in host cells by expression of antiviral proteins such as dsRNA-dependent protein kinase (PKR), 2’5’ Oligo adenylate synthetase (OA synthetase), RNAse-L, Myxovirus resistant proteins (MxA, MxB), P200 proteins and IRFs. These proteins inhibit steps in the viral life cycle i.e. penetration, replication, assembly and release of virions. Another property of type I IFNs is the anti-proliferative and anti-angiogenic functions, which are successfully applied in the treatment of cancers. Type I IFNs modulate immune function at different levels: stimulation of cytotoxic activity in T cells, NK cells, monocytes, macrophages and dendritic cells, by up regulating of MHC-I and down regulating MHC-II. Control of apoptosis by type I IFNs has been implicated as another critical biological function, which can be both pro-apoptotic and anti-apoptotic directions [Reviewed in 65, 68].

![Figure 3: Type I interferon synthesis and cellular response](image-url)
1.2.2 Interferon-beta

1.2.2.1 Immunomodulatory effects of rIFN-β in MS

As described in section 1.2.1.2, IFN-β exerts its biological functions by regulating the expression of ISGs. Of these known functions, the immunomodulatory function is considered to be most relevant for the therapeutic action in MS. Several lines of evidences indicate how IFN-β modulates the immune cells and their functions in MS.

1.2.2.1.1 Effects on immune cells:

IFN-β inhibits T-cell activation by counteracting IFN-γ by competing with the common transcription factors during IFN signaling events. IFN-γ up regulates MHC class II molecules, which is essential for antigen presentation [72]. Similarly, IFN-β may have effects on co-stimulatory signaling [73].

Administration of IFN-β in MS alters the levels of leukocyte populations in peripheral blood in a time dependent manner. The most prominent effects have been observed in natural killer cell (NK) population and their activity, while specific lymphocyte levels were also altered [74, 75].

1.2.2.1.2 Effects on cytokines:

IFN-β alters the expression of several cytokines. These include production of IL-10 [76] from mononuclear cells both at mRNA and protein levels and decreased production of IL-12, IFN-γ and TNF-α [77]. Thus, IFN-β promotes anti-inflammatory response by deviating Th1 cytokine response towards a Th2 response.

1.2.2.1.3 Effects on blood brain barrier:

A rapid decrease in gadolinium enhancing lesions can be observed in MS patients treated with IFN-β. This suggests restoration of a damaged BBB in MS. Experimental evidence indicates that IFN-β inhibits proteolytic enzymes such as matrix metalloproteinase-9 (MMP-9) and enhances the effects of tissue inhibitors of metalloproteinase-1 (TIMP-1) secreted from activated T cells [78, 79]. Similarly, IFN-β reduces the immune cell adhesion and trafficking through BBB by shedding vascular cellular adhesion molecules (VCAM) [80] from endothelium into soluble form and also reducing the integrin expression in lymphocytes [81, 82].

1.2.2.2 Early interferon trials in MS

The concept of viral infections in MS etio-pathogenesis appeared to be the idea behind IFNs as a potential therapeutic agent. During these periods, there were reports indicating defective IFN synthesis and response in MS patients, which pointed to a second rationale for IFN treatment in MS [83, 84]. The source of IFNs available for experimental work was mainly from leukocytes (IFN-α) and fibroblasts (IFN-β) cultures. When Cantell and Harvonen [85] described a method for large-scale natural IFN-α production, sufficient quantities were made available even for clinical trials including MS. One of the earliest recognizable IFN-α trials that have been reported from the work of Fog and co-workers was in 1980. They administered natural IFN-α intramuscularly for 6 progressive MS patients for 15 months, but the investigators were unable to observe clinical benefits [reviewed in 86]. Jacobs and co-workers observed decreased relapse
rates in RRMS patients following intrathecal administration of natural IFN-β [87]. A subsequent randomized, double blind and placebo controlled study confirmed their initial observation [88]. Intramuscular administration of lymphoblastoid IFNs i.e. a mixture of type I IFNs, in chronic progressive MS patients in a randomized placebo controlled study revealed no clinical and MRI evidence of improvement of the disease [89]. With these studies, natural type I interferons were proven to be beneficial mainly in RRMS [Reviewed in 86].

In late 1970s, the advent of recombinant DNA technology enabled highly purified recombinant interferons (rIFNs) to be synthesized in large scale. One of the first human proteins to be cloned was rIFN-α, but its application in MS was shown to be ineffective even though the natural IFN-α had proved promising results [90].

rIFN-γ was also tried in MS and was associated with worsening of the disease [91]. However this observations gave further opportunity for type I IFNs to be investigated in MS due to the antagonistic immunological effects between type-I and IFN-γ [Reviewed in 86].

Thirteen years of clinical trials and advancement of knowledge of the therapeutic application of IFNs in MS finally came to a success in 1993 when the first recombinant IFN-β preparation was approved for treatment in RRMS.

### 1.2.2.3 Interferon-beta pivotal clinical trials

#### 1.2.2.3.1 IFN-β1b

In 1986 a rIFN-β1b (Betaseron; Berlex Laboratories Inc, Wayne, NJ) pilot study was started to explore the optimum dose, and later extended into the large-scale multi center, double blind, placebo-controlled phase III trial comparing two doses of Betaseron (1.6 and 8 million units) in 372 RRMS patients. The study was conducted by the Multiple Sclerosis Study Group and the University of British Columbia MS/MRI analysis group across eleven universities in USA and Canada. The first interim analysis by the second year reported 33% reduction in relapse rates, which was the primary outcome measure of the trial, and longer relapse free periods in high dose group compared to placebo group. In addition, a significant decrease in MRI activity (T-2 ‘disease burden’) was also reported. However, disability progression was not significantly sustained even though scores were lower in treatment group. Well-defined side effect profiles, hematological and biochemical abnormalities could be documented during the trial. The most common side effect was flu like symptom, which was associated with the onset of therapy, and mild intermittent lymphopenia and thrombocytopenia were also observed. Based on these trial outcomes, in July 1993, the American Food and Drug Administration approved Betaseron for treatment in RRMS. It was believed that these beneficial outcomes were associated the potential ability of IFN-β to change the clinical course in MS. Accordingly, Betaseron became the first drug in a unique class of therapies in MS, the disease modifying drugs (DMDs) [92-94].

#### 1.2.2.3.2 IFN-β1a

The Multiple Sclerosis Collaborative Research group (MSCRG) started randomized, double blind and placebo-controlled phase III clinical trial with recombinant IFN-β1a (Avonex; Biogen Inc, Cambridge, MA, USA) in 1990 to determine the efficacy of first glycosylated form of IFN-β presumed to be highly homologous to natural IFN-β [95]. Three hundred and one RRMS patients with baseline EDSS 1-3.5, were enrolled in the study to receive 30 µg of weekly intramuscular
IFN-β1a or placebo for 2 years. The primary outcome measures were sustained disability progression along with relapses frequency and MRI parameters. This study reported a significant delay in time to sustained disability progression by 37% and a significant decrease in relapse rates (31%) and gadolinium enhancing lesions on MRI compared to placebo. Clinical and MRI outcome changes were comparatively lower than IFN-β1b [95].

The PRISMS study group (Prevention of Relapses and disability by IFN-β1a Subcutaneously in Multiple Sclerosis) launched an IFN-β1a (Rebif) randomized, double blind and placebo-controlled phase III trial enrolling 560 RRMS patients in 22 centers in nine countries [96]. Their rationale was that previous clinical trials had shown the efficacy of IFN-β, but that the clinical benefit yet remained uncertain and the dose of IFN-β was not optimized. They also argued that previous IFN-β1a trial had addressed a lower range of EDSS i.e. 1-3.5, which reflected rather neurological impairment than true disability. Moreover, clinical and MRI benefits were comparable to the IFN-β1b trial. Considering these pitfalls, PRISMS group included more intense dose regimen (22µg and 44µg) with subcutaneous IFN-β1a three times a week, and claimed to have employed a well-defined outcome measurement protocol. Results indicated 27-33% reduction in relapses, a significant increase in time to sustained functional impairment and disability progression and improvement in MRI parameters, especially with the high dose.

1.2.3 Antibodies to interferon-beta

1.2.3.1 Structure of the antibody

Antibodies are a class of molecules found in the gamma globulin fraction of serum. They are mainly confined to body fluids but also expressed on B-cell surfaces. Activated B cells, the plasma cells, synthesize and secrete antibodies in response to different antigenic encounters, which range from self-antigens to foreign antigens [97].
1.2.3.2 Humoral immune response

Circulating naïve B cells have surface bound IgM or IgD antibodies that act as receptors for antigens. Following antigenic contact, these B cells become activated, clonally expanded and then proceed in two pathways: either with T-cell help; the process is called T-cell dependent antibody response or without T-cell help; the T-cell independent antibody response. T-cell dependent antibody response includes several characteristics features: most antigens are proteins, antibodies are subjected to isotype switching and affinity maturation, secondary immune response is induced and memory B cells are formed. In contrast, T-cell independent responses involve direct cross-linking of antibodies on the cell surface with larger polymeric antigens such as polysaccharides, glycolipids and nucleic acids to activate B cells. Isotype switching, affinity maturation and secondary immune responses are little or none in T-cell independent responses. Thus, T-cell dependent antibody response leads to a strong and long-lived humoral immune response compared to the T-cell independent process [97].

After activation, B cells differentiate into plasma cells that synthesize antibodies. A population of memory B cells home into the bone marrow where they stay as long-lived plasma cells. The memory B cells respond promptly to subsequent antigens with an intense IgG response. The plasma cells that home into bone marrow produce constant low levels of antibodies [97].

1.2.3.3 Immunogenicity of recombinant human proteins

IFN-α is a prototype biopharmaceutical that provided firsthand knowledge in clinical applications and in the related issues of IFN treatment in human diseases [98]. Both natural and recombinant IFN-α has been used to treat several malignancies and viral infections. Reported incidences of NAbs to IFN-α are variable and several factors have been implicated in antibody development such as source of IFN, dose, route, treatment duration and type of disease [99, 100]. Diminution of biological and clinical effects are known due to NAbs in IFN-α treatment in hematological malignancies such as hairy cell leukemia [101-103] and in hepatitis-C infection [104].

A number of injected proteins used as therapeutic agents in human diseases, have shown to be associated with development of antibodies. Long before the therapeutic application of recombinant proteins, use of animal insulin to treat diabetes mellitus had been associated with insulin-induced antibodies that reduced the pharmacological effects of insulin. Improved purification methods and switching into recombinant insulin have significantly reduced the incidence of insulin-antibodies. Other examples include recombinant human growth hormone (rhGH), granulocyte macrophage colony stimulating factor (GMCSF), erythropoetin (rhEPO) and clotting factors. rhGH has higher incidence of antibodies mainly against the impurities of preparation, and therefore these antibodies were considered be of non-neutralizing type. GMCSF, rhEPO and clotting factors have shown to be typically associated with the development of NAbs, which lead to significant clinical deterioration [105].

These evidences indicate that therapeutic antibodies are critical when the clinical efficacy of a particular agent is affected. The subset of antibodies that is important in this regard is NAbs, while binding antibodies (BAbs) and antibodies to impurities have little or no clinical significance.
1.2.3.4 Anti-interferon binding and neutralizing antibodies

Healthy persons have naturally occurring auto-antibodies against IFN-α (1-10%) and IFN-β (<0.1%) and these sera have shown to be suppressing the antiviral activity of IFNs [106].

There are different viewpoints regarding the definition of different subsets of anti-IFN-β antibodies. The widely used definition is that NAbs are a subset of BAbs and are functionally important. BAbs are directed to immunogenic epitopes of IFN-β that are not involved in receptor binding, whereas NAbs bind to the areas on the IFN-β molecule that binds to the receptor (figure 5). It has been shown that appearance of BAbs precedes NAbs and the incidence of BAbs is higher i.e. 50-80% than for NAbs [107]. IgG subclass differences have been observed between BAbs and NAbs; BAbs seems to be more of the IgG1 subclass while NAbs are often of the IgG2 and IgG4 subclasses [108]. Currently, the functional relevance of BAbs is debated.

Bendtzen and colleagues [106] argue that NAb assay sensitivity determines the level of NAb detection. The amount of rIFN-β added to the assay is one of the major factors that determine the assay sensitivity. Low concentrations of rIFN-β in the assay increase the assay sensitivity while high levels of rIFN-β decrease the assay sensitivity. On the other hand, most of the BAb assays tend to have a high rate of false positive results. Thus, there is a tendency for neutralizing and binding antibody results to be non-parallel. They argue that such a discrepancy may have contributed to the conception of two subsets of anti-IFN-β antibodies; the BAbs and NAbs. With a series of validation experiments, they have optimized both NAb and BAb assays concluding that specific BAbs are probably always NAbs [109]. Further to this, they point out that when an antigen-antibody complex is formed, the differences in molecular size between IFN-β and Ig (NAbs or BAbs), i.e. Ig molecules are approximately 150,000 in molecular weight whereas IFN-β is about 20,000, oblitative effects to IFN-β and interferon receptor interaction is likely to occur regardless of epitope, leading to non-activation of IFN signaling; the neutralizing effect of IFN-β.

![Figure 5: Neutralizing and non-neutralizing antibodies](image)

1.2.3.5 Observations of anti-IFN-β NAbs in pivotal clinical trials

1.2.3.5.1 Early IFN trials

Use of fibroblast interferons (natural human IFN-β) to treat nasopharyngeal carcinomas was associated with development of serum neutralizing antibodies. Such IFN-β was produced from the same patient’s fibroblast cell line, i.e. supposedly immunogenically endogenous protein. Thus a question was raised whether these antibodies were autoantibodies against human IFN-β. This was one of the first reports on development of anti-IFN-β neutralizing antibodies in a homologues
system [110, 111]. Likewise, serum-neutralizing effects were observed in several studies especially in early trials with MS patients.

1.2.3.5.2 Observations of NAbs in IFN-β1b trial

At the conclusion of phase III IFN-β1b trial in 1993, 45-47% IFN-β1b treated patients were reported to have developed NAbs [92]. After 3 years, the study group presented a systematic account on immunogenicity of IFN-β1b in 1996 following properly defined protocol for NAb analysis [112]. The viral cytopathic assay was used to test NAbs in serum samples obtained 12-36 hours after last IFN-β injection. The cut off level for NAb test positivity was defined as neutralizing titer ≥ 20 based on the observation of the placebo group’s NAb results to exclude false positives. By about the 6th month of IFN-β treatment, 15-19% of the patients became NAb positive and the incidence peaked at second year by 38-40%. An effect of NAbs on relapse rates was observed after about 18 months in the high dose group (8 MIU). Relapse rates, numbers of new lesions and enlarging lesions were higher in the NAb positive group compared to that of NAb negative patients. They observed a non-influential effect on EDSS, which is seemingly obvious because Betaseron was proved to be non-effective on EDSS in the original trial. Thus, it was clearly documented that a significant percentage of patients do develop NAbs to IFN-β and, more importantly, they tend to have decreased clinical efficacy due to NAbs [92, 93, 112].

1.2.3.5.3 Observations of NAbs in IFN-β1a trial

The pivotal phase-III trial evaluating efficacy of intramuscular IFN-β1a (Avonex) also reported the prevalence of NAbs at different time points such as 14% by week 52, 21% by week 78, and 22% by 104 [95]. This preliminary report was followed by a further analysis to evaluate the biological and clinical significance of NAbs [113]. Serum was tested for BAbs, NAbs and in vivo bioactivity using serum Neopterin and β2-microglobulin assays from phase-III trial and phase-VI extension studies. The overall NAb positivity was reported as low as 5% compared to the previous analysis. However, the reason for this low seroprevalence in the new analysis is not known, but it is claimed that the patients had been receiving an improved commercial form of the drug during the extension phase of the trial. However, NAb levels peaked at 18 months (4-10%) followed by a gradual decrease after 24 months (1-6%). It was observed that the in vivo bioactivity was diminished with NAbs. No differences were observed in relapse rates or disability progression except an increase in gadolinium enhancing lesions in NAb positive patients. Thus, they demonstrated an effect of NAbs on the biological activities of IFN-β1a in MS and some MRI deterioration. In addition, they observed a very high prevalence of NAbs in IFN-β1b (39%) compared to IFN-β1a (6%) [114]. European IFN-β1a Dose Comparison group reported higher relapse rates (12-48 months after treatment), EDSS progression (by 48 months) and increased MRI activity (24-36 months) after NAb development in a four-year immunogenicity study [115]. The overall conclusion was that clinical effects of NAbs were likely to be dependent on titer level and the persistence of NAb. They further recommended that given the cost of therapy and the inherent difficulty in monitoring for clinical response, the treatment should be discontinued if NAbs are persisting.

Similarly, NAbs to subcutaneous IFN-β1a (Rebif) were observed in PRISMS pivotal phase III trial at the conclusion of the second year [96]. Of the two dosage arms of Rebif, 23.8% of patients developed NAbs in 22µg group and 12.5% of patients in 44µg group. During the extension phase (PRISMS-4), the prevalence of NAbs was 14.3% and 23.7% in patients treated with 44µg and 22µg respectively after 4-year period. The effect of NAbs on clinical efficacy was assessed both
at the end of phase III and at the end of the 4-year extension study. A significant increase in relapse rates and MRI parameters were observed in NAb positive group compared to NAb negative group especially after 2-3 years of treatment onset, concluding that development of NAbs have significant impact on previously observed beneficial outcomes of therapy [96, 116].

1.2.3.6 Observations in other studies

A nation-wide Danish study was done with 541 MS patients who were tested for NAbs by using the CPE assay at yearly interval for 5 years. Increased relapse rates and time to first relapse, but no change in EDSS was observed in those who were positive for NAbs. They pointed out that detection of NAbs and thereby the frequency of NAbs was highly dependent on sensitivity of the NAb assay [117].

1.2.3.7 Factors influencing development of NAbs

As for most injectable bio-pharmaceuticals (see section 1.2.3.3), factors such as type of product, dose, route, frequency, treatment duration, type of assay and miscellaneous patient factors have been implicated for development of NAbs to IFN-β in MS.

a. Commercial preparation

Incidence of both BAbs and NAbs vary among different IFN-β preparation [95, 113, 117, 118]. Avonex has the lowest NAb frequency while Betaferon/Betaseron has the highest frequency of NAbs. rIFN-β1b is produced in Escherichia coli, whereas IFN-β1a is produced from mammalian cell lines. There are differences in the amino acid sequence of IFN-β1b compared to natural IFN-β. There is a cysteine-to-serine substitution at position 17 and a deletion of a N-terminal methionine residue. IFN-β1b is non-glycosylated while IFN-β1a is glycosylated with more resemblance to the endogenous IFN-β [Reviewed in 118]. These major differences in structure have been frequently attributed to the differences observed in NAb frequency. The non-glycosylated form is less soluble and tends to form aggregates, which are presumed to be responsible for the highest NAb frequency observed for IFN-β1b. On the other hand, different IFN-β1a preparations have also shown differences in frequency of NAbs, which is thought to be due to the differences in tertiary structures during the post translation modification process in the manufacturing process [119]. Table 1 lists the pharmacological differences between rIFN-β preparations [120].

b. Dose

High dose of IFN-β has been associated with high incidence of NAbs, which was evident from the comparison trials of Avonex (30µg and 60µg intramuscularly, once a week) and Rebif (22µg and 44µg once a week subcutaneous) [121, 122]. However, the standard regimens of Rebif (22µg and 44µg 3 times a week subcutaneous) had inverse dose relationship with respect to the incidence of NAbs [96, 116]. The reason for this observation is not clear, but is thought to be due to the reduced assay sensitivity by the presence of serum IFN-β or to the induction of tolerance [109]. As can be observed in table 1, the weekly protein dose is higher in the IFN-β1b regimen than IFN-β1a, which could be another reason for high incidence of NAbs in IFN-β1b patients.
c. **Route**
The effect of the route of administration on NAb development is still unclear. All three preparations have major differences in dose and frequencies rendering difficulties to make direct comparisons. Perini and co-workers compared the Betaferon standard regimen with Betaferon administered intramuscularly once a week. This showed higher incidence of NAbs in the subcutaneous route [123]. However, the differences in the frequency of administration may influence the results.

d. **Frequency**
Low dose (22µg) Rebif given once a week versus 3 times a week indicated a higher incidence of NAbs in the more frequent regimen [96, 109].

e. **Antibody assay**
The incidence of NAbs varies depending on the type and the sensitivity of the NAb assay [109]. There is no standardized NAb assay, which produce consistent results and therefore, different labs have different protocols despite the effort of bringing a consensus on inter laboratory assay validations [124].

**Table 1:** Comparison of three commercial IFN-β preparations

<table>
<thead>
<tr>
<th>Source</th>
<th>IFN-β1b</th>
<th>IFN-β1a</th>
<th>Rebif</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source</strong></td>
<td>Betaferon/Betaseron</td>
<td>Avonex</td>
<td>CHO*</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>18.5</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Specific activity (MIU/mg)</td>
<td>32</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>Dose (µg)</td>
<td>250</td>
<td>30</td>
<td>22 or 44</td>
</tr>
<tr>
<td>Frequency</td>
<td>every other day</td>
<td>weekly</td>
<td>thrice weekly</td>
</tr>
<tr>
<td>Route of administration</td>
<td>subcutaneous</td>
<td>intramuscular</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>Weekly dose (µg/week)</td>
<td>875</td>
<td>30</td>
<td>66 or 132</td>
</tr>
</tbody>
</table>

CHO* - Chinese hamster ovarian cells

This evidence suggests that seroprevalence (NAb positive frequency in a patient group) differs among IFN-β preparations and that this is influenced by several factors. NAbs to IFN-β1b appears to be more seroprevalent than those to IFN-β1a. Frequent administration by subcutaneous injection is seemingly a potential antigenic route compared to less frequent intramuscular route. Binding antibody frequencies also follows a similar pattern with regards to IFN-β preparations, dose, and route of administration [109].

1.2.3.8 **NAbs and IFN-β bioactivity**

Since NAbs block IFN-β-receptor engagement, the downstream signaling pathways are not activated or only partially activated giving rise to variable expression levels of antiviral mRNA or proteins. This principle has been applied for the development of biomarkers such as MxA, neopterin, β2-microglobulin and 2,5 OAsynthetase to monitor in vivo response of IFN-β [125, 126]. Of these, MxA is considered a both sensitive and specific surrogate marker for IFN-β bioactivity [126, 127] and thereby, it has been identified as the most appropriate biomarker to develop bioassays [125, 128] and to monitor therapeutic response to IFN-β [129, 130].
Deisenhammer and co-workers compared bioactivity in patients treated with different IFN-β preparations (NAb negative) and they found that the subcutaneous IFN-β1b was giving the highest in vivo bioactivity response measured by MxA protein levels in peripheral blood leukocytes [131]. A similar observation was made in an Italian study in which both subcutaneous IFN-β1a and IFN-β1b gave higher bioactivity response compared to intramuscular IFN-β1a [130].

Development of NAbs and interference by NAbs with IFN-β bioactivity has been observed ever since the pivotal clinical trials [113, 116] and subsequently by several independent studies [107, 128, 132, 133]. This phenomenon is known as antibody-mediated decreased bioactivity (ADB) and its measurement has been recommended to assess neutralizing effect of NAbs in serum [125, 134]. Since there is no effective clinical or MRI surrogate markers to evaluate treatment response to IFN-β and hence to assess the effects of NAbs in MS directly, bioactivity measurements offer a logical and a more practical alternative [135].

Several studies have reported that there is a negative correlation with IFN-β bioactivity and NAb levels as mentioned above [107, 113, 116, 128, 132, 133]. However, this has often been reported at the patient group levels. There is less clear evidence on the precise levels of ADB although high NAb titers have consistently been associated with pronounced inhibition of IFN-β bioactivity [113, 125, 136].

One of the deficiencies in our understanding of NAbs is the relationship between the bioactivity and the NAb titers at the individual patient level. The second study (paper II) in this thesis addressed this question with the following hypothesis.

a. There would be a titer dependent inhibition of bioactivity, which is measurable in terms of in vivo MxA expression levels.
b. Thus, the MxA expression levels would be helpful to define functionally relevant NAb titer levels that are useful in a clinical setting.

1.2.3.9 Development and fluctuation of NAbs

NAbs are usually detectable during the first year of treatment and as early as at the third month after onset of therapy depending on the assay sensitivity [109]. In a majority (50-60%) of patients who develop NAbs against IFN-β1b, these tend to disappear spontaneously over time (~20 months). This has been observed in the pivotal clinical trial [112] and by several independent studies [137, 138]. However, high titers of NAbs tend to persist for a longer period [138, 139]. Similarly, seroconversion to negativity has also been observed in treatment with IFN-β1a. A Danish study that evaluated the persistence of NAbs by following up 455 MS patients, reported that if a patient is negativity for 24 months or positive for 18 months, these NAbs status tend to be persistent [138]. Thus, basically the type of IFN-β preparation and the titer determines the evolution of NAbs.

In study III, we hypothesized that the characterization of NAb titer fluctuation in a large sample material in a clinical setting would be useful in prognosticating future NAb status in a given patient.
1.2.3.10 Consensus and controversies on clinical importance of NAbs

As stated above, a negative clinical impact of NAbs on ongoing IFN-β treatment has been observed in major controlled clinical trials and in independent studies [93, 96, 112, 115, 116]. In 2004, a panel of expertise from European Federation of Neurological Society (EFNS) reached a consensus after revising the available literature. They concluded that the IFN-β NAbs are a clinically important entity and the necessity for periodic NAb testing and suggested a management of those who develop NAbs [124]. Their recommendations included:

- Screening for BAbs followed by NAb test for those who are positive for BAbs.
- Usage of validated CPE or MxA induction assay to measure NAbs and calculation of NAb titer using the Kawade method.
- Testing all patients for NAbs by 12-24 months after IFN-β treatment and those who develop NAbs should be re-tested after 3-6 months intervals.
- IFN-β treatment should be discontinued if high NAb titers are persistent.

However, there is some skepticism among the others; the Evidence report of Therapeutic and Technological Assessment Subcommittee of American Academy of Neurology [140] claimed that the association between NAbs and reduction in clinical and radiological effectiveness of IFN-β treatment is rather a probable relationship. They argued that the disappearance of NAbs over time makes the clinical importance of NAbs controversial and that there is insufficient information to provide recommendations on when to test, which test to use, how often to test and what titer cut off level to be applied for NAb testing [140].

However, some of these viewpoints warrant a critical re-visiting and analysis of these reports while appreciating the controversial nature of the issue. The different time relations of appearance of NAbs, affinity maturation and hence the timing for negative impacts of NAbs has been implicated as the basis for these prevailing controversies [141]. In addition, in a recent report, Goodin and co-workers observed a low prevalence of NAbs in a large sample of IFN-β treated MS patients who were supposed to be worsening in spite of treatment. Their sample was the largest ever examined and their observations thus added to the controversy on the clinical importance of NAbs during the IFN-β treatment [142]. The study IV in this thesis was performed to examine a similar question in our sample material by a cross sectional analysis.
2  AIMS OF THE THESIS

Recombinant Interferon beta is currently the most wide-spread immunomodulatory treatment in MS. Even though new therapies are emerging, the superior safety profile that has been experienced with IFN-β over the last two decades is impressive. However, one of the major clinical issues that was encountered as early as in the pivotal clinical trials is the development of antibodies to IFN-β, of which, the subset of neutralizing antibodies (NAbs) gained much interest among clinicians, patients and scientists due to the fact that NAbs appear to interfere with clinical efficacy of the drug.

The overall aim of this thesis was to investigate some aspects related to NAbs in MS in order to provide evidence-based knowledge useful for clinical management of MS patients.

The specific aims were as follows:

STUDY I
To assess NAb seroprevalence (frequency of patients with NAbs) and immunogenicity (titer levels) of IFN-β preparations in a clinical setting.

STUDY II
To determine how NAb titers interfere with the in vivo bioactivity of IFN-β especially at the individual patient level and thereby, to identify functionally critical NAb titer levels to determine clinically useful NAb titer categories.

STUDY III
To identify the pattern of NAb titer fluctuation over time and hence to predict future NAb status and suggest NAb testing intervals.

STUDY IV
To determine whether there is an association between NAb positivity and the reported reasons for NAb testing, i.e. to investigate whether NAb status can be predicted on clinical grounds.
3 MATERIALS AND METHODS

3.1 PATIENTS AND CONTROLS

Serum samples from MS patients were sent from neurology units in Sweden, Norway and Finland to our specialized NAb testing laboratory in the Division of Neurology, Karolinska University Hospital Huddinge. A referral form (see appendices), which included information on demographic data of patient, diagnosis, currently prescribed IFN-β preparation, duration of treatment, and indication for NAb testing was also sent with the serum samples. IFN-β is indicated for the treatment of patients with RRMS, but also for patients in early SPMS who are still experiencing relapses.

Study I included 1,115 consecutive first time serum samples from MS patients that had been referred from 2003 to 2004. Of these patients, 113 patients had repeated samples and, thus, only their first samples were included in the study. Seropositivity, NAb titer levels of different IFN-β preparations and treatment durations were assessed retrospectively.

In study II, 97 MS patients who had been previously tested for NAbs, were enrolled. All were on one of the IFN-β preparations and had variable treatment durations. They were selected to offer a gradient of NAbs from negative to very high NAb titers. Ten healthy controls and 13 MS patients who had never been treated with IFN-β were included as controls. These untreated MS patients subsequently received IFN-β treatment and were then included in the NAb negative control group with a subsequent sample. Peripheral venous blood samples were collected from all MS patients and healthy controls to determine *in vivo* IFN-β bioactivity by MxA mRNA assay.

Study III included 822 IFN-β treated MS patients who had been tested for NAbs at least twice during the period of 2003 to 2007. These patients were on different IFN-β preparations and had variable treatment durations. The blood sampling intervals spanned between 1 and 3 years. The difference in NAb titers between first and last samples was analyzed in the context of treatment duration and sampling interval.

Study IV included 2,822 first-time serum samples for NAb testing from MS patients referred to our NAb testing laboratory from 2003 to 2006. Of these samples, 2,506 referrals included information on reason for NAb testing, and were subjected to the main data analysis.

Informed consent was obtained from patients for participating in research and the regional ethical committee in Stockholm approved the work.
3.2 MEASUREMENT OF NEUTRALIZING ANTIBODIES TO INTERFERON-BETA

MxA induction assay was used to screen and to titrate the NAbs in patient’s sera. The serum was separated from peripheral venous blood collected at least 48 hours after the last IFN-β injection. Serum samples were frozen before they were sent to the NAb testing laboratory.

In study II, an additional peripheral venous blood sample was collected into PAXgene blood RNA tubes (PreAnalytix GmbH, Hombrechtikon, CH) from selected patients and from controls for in vivo MxA mRNA assay, as described in section 3.2.2.

3.2.1 MxA induction assay

The MxA induction assay was described by Files and Pungor [143, 144] and was based on the original work by Yoshimi Kawade and co-workers [145]. The protocol has been described in detail in paper I and briefed in paper II, III and IV of this thesis.

The MxA assay is based on measurement of IFN-β bioactivity either by MxA protein or MxA mRNA expression in a cell line (A549 lung carcinoma cells -American type tissue culture) challenged with rIFN-β (Avonex) in the presence of patient’s serum. If the serum contains NAbs to IFN-β, the free IFN-β is blocked. This will reduce IFN receptor activation and biomarker expression. The obtained neutralizing potency is defined as a ten-fold reduction of IFN-β bioactivity.

Practically, this is achieved in two stages. Firstly, an IFN-β standard curve is generated by serially diluting antigen (rIFN-β) starting from 10LU/ml. MxA expression is measured and plotted against the log values of the IFN-β dilution series. Then the MxA level corresponding to 1LU/ml, which is known as EC₅₀ in a typical sigmoidal IFN-dose response curve (the standard curve) is determined (Figure 6, left). There is inherent biological variation in the assay and therefore, the standard curve is validated at an EC₅₀ level with an acceptable value around 1LU/ml. Secondly, a patient’s serum is serially diluted in the presence of a fixed amount of rIFN-β (10LU/ml) and the point of serum (antibody) dilution that reaches the MxA expression level corresponding to EC₅₀, is determined (figure 6, right). The NAb titer derived at this stage is termed the “raw” or “unadjusted” titer (f).

![Figure 6: The methodological principle of MxA induction assay](image)

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With a given antibody concentration in the neutralization reaction, there is a ratio (n) between total IFN (I_t) and remaining IFN (I_f) in the solution as described by Kawade et al. The raw NAb titer (f) can be adjusted using the formula suggested by Kawade and expressed as ten-fold reduction units/ml (TRU/ml) and termed the Kawade titer:

\[ t = f \cdot (n-1) / (10-1) \]

\( t = \) neutralizing titer, \( f = \) reciprocal of serum dilution, \( n = \frac{\text{total IFN-}\beta}{\text{residual IFN-}\beta} \)

Thus, NAb titers obtained by neutralizing assays by different investigators should be comparable to each other when titers are adjusted according to Kawade and expressed as TRU/ml. A majority of routine NAb analyzing laboratories in Europe and in USA use this correction. Since this is a cellular bioassay, it has an inherent interassay variability. We assessed this in a separate experiment by measuring NAbs in 50 serum samples twice at different time points. The interassay variability was found to be ranging from 1% to 25% with a mean of 5%.

**NAb titer categories:**

In all studies, NAb positivity was defined as ≥10 TRU/ml. In paper I, the following NAb titer categories were used during data analysis:

- **Negative**: <10 TRU/ml
- **Low**: 10-49 TRU/ml
- **Medium**: 50-199 TRU/ml
- **High**: 200-1280 TRU/ml
- **Very high**: >1280 TRU/ml

Once the IFN-\(\beta\) bioactivity data was available after study II, the above titer categories were redefined as follows. Thereby, the rest of the analyses in studies II, III and IV were done using the redefined NAb titier categories.

- **Negative**: <10 TRU/ml
- **Low**: 10-49 TRU/ml
- **Medium**: 50-149 TRU/ml
- **High**: 150-599 TRU/ml
- **Very high**: >600 TRU/ml

### 3.2.2 MxA in vivo mRNA assay

An alternative approach to determine neutralizing effect in IFN-\(\beta\) treated MS patients is to measure the in vivo bioactivity of IFN-\(\beta\) after administration of the drug [107]. Systemically administered IFN-\(\beta\) activates IFN receptors and downstream signaling pathways in cells, including leukocytes. Presence of NAbs thus interferes with IFN receptor activation and reduces the bioactivity of IFN-\(\beta\), which can be assessed either as soluble MxA protein in the blood [107] or by MxA mRNA expression [125, 146] in peripheral venous blood cells.
In the second study, IFN-β bioactivity was measured by MxA mRNA expression level by using reverse transcriptase real-time polymerase chain reaction (RT-PCR). A detailed methodology of this method is described in paper II.

3.2.2.1 Methodology of MxA in vivo mRNA assay in brief:

Peripheral venous blood (2.5 ml) was collected into PAXgene (PreAnalytix GmbH, Hombrechtikon, CH) blood RNA tubes that stabilize whole blood RNA instantly as the blood is drawn into the blood tube. The RNA quality and integrity was assessed by capillary electrophoresis, using Agilent Bioanalyzer (Agilent Technologies, GmbH, Waldbronn, Germany), to evaluate the RNA stabilizing effects of the PAXgene buffer at different storing conditions (figure 7). The PAXgene system appears to be stabilizing the whole blood RNA and yields RNA with good quality and integrity both in ambient and frozen temperatures during transport or in subsequent storage stages.

<table>
<thead>
<tr>
<th>Blood samples</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1 &amp; 2</td>
<td>2 hours at room temperature</td>
</tr>
<tr>
<td>Sample 3 &amp; 4</td>
<td>4 days at room temperature</td>
</tr>
<tr>
<td>Sample 5 &amp; 6</td>
<td>2 weeks at –20 °C</td>
</tr>
<tr>
<td>Sample 7 &amp; 8</td>
<td>4 weeks at –70 °C</td>
</tr>
<tr>
<td>Sample 9 &amp; 10</td>
<td>1 year at –70 °C</td>
</tr>
</tbody>
</table>

Figure 7: Capillary electrophoresis of total RNA extracted from peripheral venous blood. Gel-like image (top left), electrophoretic data (right) and the storage conditions are shown. All samples show distinct 18S and 28S ribosomal bands which indicate intact RNA.

Total RNA was extracted from blood samples and treated with DNase (Qiagen GmbH, Hilden, Germany) to minimize genomic DNA contamination. Total RNA was subsequently reversely transcribed into cDNA (Taqman® reverse transcription kit, Applied Biosystems, Roche Molecular Systems Inc., Branchburg, New Jersey, USA) according to the manufacturer’s protocol. The 9600 GeneAmp PCR system was used for RNA extraction (Applied Biosystems, Norwalk, CT, USA).
Taqman® real-time PCR system (ABI PRISM 7700 Sequence Detector, Taqman® universal PCR master mixture and protocol, Applied Biosystems, Foster City, CA, USA) was used to determine MxA (target gene) and GAPDH (endogenous control) mRNA expression levels. Relative standard curve method (user bulletin #2, ABI PRISM 7700 sequence detecting system, Applied Biosystems) was used for PCR quantification.

A batch of standard curve samples was prepared by stimulating A549 human lung carcinoma cells with IFN-β1a to express high levels of MxA mRNA. Extracted RNA from these cells was converted to cDNA as described above. Aliquots of these cDNA (equivalent to 100 ng of input RNA) were batched and stored at –20°C. During each PCR assay, a fresh aliquot of cDNA from this batch was used to prepare five serially diluted samples (100, 10, 1, 0.1 and 0.01 ng) to generate both MxA and GAPDH standard curves. The MxA mRNA assay was validated by investigating the interassay variation of the PCR standard curve slopes and intercepts of 6 different runs. For MxA, the curve slope was -4.50 to -3.79 with a Y intercept of 26.63 to 28.74 and for GAPDH -3.82 to -3.33 and 22.42 to 23.59 respectively. All assays accepted had excellent correlation of curve, i.e. R^2 (table 2). These values were then used as criteria for accepting subsequent runs of the samples.

**Table 2:** Validation of MxA mRNA assay

<table>
<thead>
<tr>
<th>MxA</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Assay 4</th>
<th>Assay 5</th>
<th>Assay 6</th>
<th>Assay cut off levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>R^2</td>
<td>0.9878</td>
<td>0.9916</td>
<td>0.9932</td>
<td>0.9901</td>
<td>0.9968</td>
<td>0.9972</td>
<td></td>
</tr>
</tbody>
</table>

| GAPDH | | | | | | |
| Intercept | 23.4815 | 23.3878 | 23.5913 | 23.0793 | 22.4285 | 22.5009 | 22.4285 to 23.5913 |
| R^2 | 0.9964 | 0.9973 | 0.9966 | 0.9952 | 0.9976 | 0.9982 |

**3.2.3 Other gene expression assays**

In the study II, Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mRNA expression was also measured using RT-PCR to corroborate the in vivo MxA mRNA expression data. Relative TRAIL mRNA expression levels were correlated to MxA mRNA expression and to the NAb titer gradient.

**3.3 STATISTICS**

In studies I, III and IV, the Fisher exact and chi-square tests were used where appropriate to compare the following variables: proportions of patients in NAb titer categories, prescribed IFN-β preparation, treatment duration and blood sampling interval. The Mann-Whitney test was used to compare demographic variables i.e. age.

In study II, to compare mRNA expression levels between controls and MS patients, the Mann-Whitney test and the Kruskal-Wallis test with post Dunn correction (for multiple comparisons) were used. The Spearman correlation was performed to correlate mRNA expressions and NAb titer levels. GraphPad Prism software was used to perform statistical tests. (GraphPad Software (version 4.00), San Diego California USA, www.graphpad.com).
4 RESULTS

4.1 SEROPREVALENCE AND IMMUNOGENICITY (STUDY I)

In Study I, 1115 first-time serum samples from IFN-β treated patients were analyzed in a retrospective manner in order to assess frequency of NAb positivity, titer inducing potentials and effect of treatment duration on NAb status. Seventy five percent of all patients were females (n=833) and 25% (n=282) were males. The mean IFN-β treatment duration was 3 years and 4 months.

The overall NAb positivity was 32% (n=356). We have chosen to refer to the NAb positive frequency as seroprevalence, and suggest that the previously much used term immunogenicity should be reserved to define titer levels.

Thus, the seroprevalences observed in this study were:

1. Avonex 13% (n=34)
2. Betaferon 43% (n=125)
3. Rebif22 39% (n=108)
4. Rebif44 30% (n=89)

Thus, of all preparations, the lowest seroprevalence was observed in patients treated with Avonex (P<0.0001). The highest seroprevalence was observed for Betaferon, which was also significantly higher than Rebif44 (P=0.001). A higher seroprevalence was observed in Rebif22 compared to Rebif44 (P=0.02).

One of the important observations that we made in this study was the titer inducing potentials of different IFN-β preparations. We specifically referred this property as immunogenicity. Figure 8 illustrates the capacity of IFN-β preparations to induce various titer levels and chi-square trend analysis revealed that these trends were differing significantly between preparations. Avonex and Betaferon tended to induce low to intermediate NAb titers respectively, whereas Rebif22 and Rebif44 tended to induce high titers, i.e. showing high immunogenicity.

![Immunogenicity of IFN-β products](image)

**Figure 8:** Immunogenicity among different IFN-β preparations. X-axis represents NAb titer categories and Y-axis represents the percentage of NAb positive patients.
Effect of treatment duration on seroprevalence was analyzed in 880 referrals that had the information on treatment duration. The general impression here is the persistence of NAbS rather than the expected decrease with time. Seroprevalence was strikingly similar throughout the categories, with only a minor peak in patients treated for 3 to 5 years. Specifically, patients treated more than 6 years were just as likely to be NAb positive as those treated between 2 and 3 years.

An additional observation made in this study was that NAb positive patients tended to be older (median 45 years (21-74)) than the NAb negative patients (43 years (18-79)) and this was found to be statistically significant (p<0.001). Subsequent multivariate analysis of age in different preparations, gender and treatment durations were found to be non-significant.

4.2 FUNCTIONALLY CRITICAL NAB TITER LEVELS (STUDY II)

IFN-β bioactivity was measured by the MxA induction assay in patients with different NAb titers to identify functionally relevant titer levels.

Of the 97 IFN-β treated MS patients analyzed in this study, 14 were treated with Avonex, 20 with Betaferon, 15 with Rebif22 and 48 with Rebif44. The majority of the patients were diagnosed to have relapsing remitting MS (88) and nine patients were in secondary progressive MS. The mean EDSS score (expanded disability status scale) was 3.5. Treatment durations varied between 12 and 108 months (mean 47 months).

To define a baseline mRNA expression level for the in vivo MxA expression assay, were used the mean relative MxA mRNA expression values in IFN-β-naïve healthy controls (mean 1.23, SD 0.99) and IFN-β untreated MS patients (mean 1.17, SD 1.44) (i.e. mean+3SD=4.84). Thus the baseline level was set at MxA mRNA expression 5.

When antibody-free MS patients (negative controls) were re-sampled 12 hours after their initial IFN-β injections, a sharp rise of MxA expression (mean 33.37, SD 26.36) was observed above the baseline. Interestingly, a strikingly variable individual response was noted.

The in vivo MxA mRNA expression (bioactivity) was negatively correlated (Spearman r=-0.83, p<0.0001) with NAb titers of IFN-β treated MS patients (n=97) at a high level of significance. All IFN-β treated MS patients showed high MxA expression levels after IFN-β injection if their NAb titers were below 150 TRU/ml (mean 46.1) compared to the healthy controls, negative controls and those who had NAb titers above 150 TRU/ml. In contrast, patients in the range from 150 to 600 TRU/ml had greatly reduced MxA responses, but still clearly above the baseline (mean 9.4). Patients with NAb titers above 600 TRU/ml had a complete loss of IFN-β bioactivity, showing MxA levels close to or below the base line MxA level (mean 3.67). Thus, we observed three categories of MxA mRNA responses:

1. NAb titers up to 150 with a largely retained MxA response.
2. Low response group between 150 and 600.
3. Above 600 TRU/ml with virtually complete loss of the response.
The average timing after the last IFN-β injection was 13 hours and the majority of the patients had arrived at the clinic for blood sampling as instructed in the protocol, i.e. around 12 hours after last IFN-β injection. Correlation analysis between MxA expression and sampling time did not show any significance, but the 3 patients showing lowest MxA expression in the NAb titer range 0-150 TRU/ml had been sampled after 12 hours.

Analysis of TRAIL mRNA showed a positive correlation with MxA mRNA expression (Spearman r=0.71, p<0.0001) and a negative correlation with NAb titer (Spearman r=−0.65, p<0.0001) corroborating the observations made with MxA-based bioactivity assessment.

### 4.3 FLUCTUATION OF NAB TITER (STUDY III)

In the third study, we assessed the fluctuation of NAb titers over the time. We used the functionally critical NAb titer level 150 TRU/ml as the main border across which the fluctuation was analyzed. NAb titers that were changing across this level either from lower to a higher level or vice versa, were presumed either as losing or regaining IFN-β bioactivity respectively.

We enrolled a total of 822 IFN-β treated MS patients, who were tested for NAbs at least twice. Their sampling interval between first and second samples was 1 to 3 years (mean sampling interval was 1 year). Mean age was 45 years and 70% of the patients were females. All patients were on one of the IFN-β preparations; Avonex (18%), Betaferon (25%), Rebif22 (21%) and Rebif44 (36%) and treatment durations varied from 6 to 144 months (mean 33 months).

Overall, 72% of the patients showed NAb levels in the same titer category in their first and second samples, that is, the majority of the patients did not experience changes in NAb titers. Especially, patients who were NAb negative or had very high titers in their first samples remained negative (90%) or in the very high category (83%), respectively, in their second NAb samples. In contrast, patients showing low, medium or high titers tended to fluctuate more often into other categories.

Analysis of specific titer categories showed that if the first sample was NAb negative (n=366), the risk of loss of bioactivity over an average of 12 months was only 3%. Similarly, if the first sample showed a low (n=125) or medium (n=64) titer, the risk of loss of bioactivity was 6% and 22% respectively. The chance of regaining bioactivity among patients in the high titer (n=84) category was 27%, but only 9% if the NAb titers were very high (n=183). Thus, when a patients’ NAb titer is close to the functionally critical level i.e. 150 TRU/ml/ml, there is increasing chance of titer fluctuation.

Table 3 summarizes the chance of NAb titer fluctuations across the functionally critical titer of 150 TRU/ml/ml. Only 6% of 555 patients (mean treatment duration 27 months) who had NAb levels assumed to indicate good IFN-β in vivo bioactivity moved into a level high enough to indicate loss of response. In contrast, of those who were likely to have lost their bioactivity at first sampling (n=267, mean treatment duration 35 months), 15% were expected to have regained bioactivity when re-sampled. Overall, there was a greater chance of reverting to lower NAb levels compared with developing levels indicating loss of bioactivity and this comparison was significant (P<0.001).
Table 3: Chance of good bioactivity versus risk of loss of bioactivity

<table>
<thead>
<tr>
<th></th>
<th>First samples</th>
<th>Second samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;150 TRU/ml</td>
<td>&gt;150 TRU/ml</td>
</tr>
<tr>
<td>&lt;150 TRU/ml (n=555)</td>
<td>94%</td>
<td>6%</td>
</tr>
<tr>
<td>&gt;150 TRU/ml (n=267)</td>
<td>15%</td>
<td>85%</td>
</tr>
</tbody>
</table>

Effect of different IFN-β preparations, treatment duration and sampling interval on NAb titer fluctuation failed to show a significant confounding effect on NAb titer fluctuation when statistically analyzed.

4.4 IMPRESSION OF CLINICAL WORSENING AND NAB STATUS (STUDY IV)

This study aimed at retrospectively analyzing a large sample of NAb data from 2,822 IFN-β treated MS patients with a special focus on reasons or indications for NAb testing. Of these, 2506 referrals had information on reason for NAb testing as ‘routine’, ‘worsening of the disease’ or ‘other reasons’.

Overall, 31% of all patients were NAb positive and for each IFN-β preparation, the seroprevalences were: eleven percent for those who were treated with Avonex, 36% of those treated with Rebif and 43% of those treated with Betaferon. Seventeen percent of those who were NAb positive, showed high or very high titers. Of them, 5% were treated with Avonex, 20% were treated with Betaferon and 23% were treated with Rebif. This analysis, confirmed the findings in study I on seroprevalence and immunogenicity differences found between the different IFN-β preparations.

The reason for NAb testing was indicated on 2,506 (88%) referrals. Of these, in 14% (n=363) of instances, the indication for testing was clinical or radiological worsening of disease, for 76% (n=1,904) of the patients the indication for NAb testing was routine. Ten percent (n=239) of the patients had other reasons: test series, presence of side effects, absence of side effects, combination therapy, and consideration of another treatment.

Thirty one percent (n=112) of those who were referred with an indication of worsening of the disease) were NAb positive, whereas, 30% (n=562) of the patients who were tested routinely were NAb positive. The statistical comparison revealed no significant difference (p=0.26). Similarly, 18% of the patients tested due to worsening showed high or very high NAb titers (>150 TRU/ml) compared to 16% of the patients who were tested routinely. This difference was not statistically significant (p=0.56). Analysis at each NAb titer category levels also revealed non-significant comparisons for reasons for NAb testing, even after stratification into different treatment durations.
Betaferon treated patients were more often tested for an apparent clinical worsening compared to patients treated with Avonex (p=0.05) and patients treated with Rebif (p=0.026). Patients treated with Avonex were more often tested routinely than patients treated with Betaferon (p=0.0008) and patients treated with Rebif (p=0.029).

Patients with a sampling indication of worsening were younger than patients with other indications for testing (p=0.023). Patients with “other indications” for NAb testing had longer duration of treatment than patients with worsening indicated (p=0.001) and patients with a routine indication (p=0.0001).
5 DISCUSSION

Biopharmaceuticals are one of the essential classes of therapeutics for a number of human diseases in modern-day clinical medicine. Development of antibodies against these biopharmaceuticals is an expected problem, because most often these are proteins or biopolymers, which are potentially immunogenic to the host immune system and have a capacity to mount immune responses. Of all cytokines that are known today, interferons were the first to be purified, cloned, sequenced completely, and produced in a recombinant form. Most importantly, interferon was the first cytokine to reach a point of widespread clinical applications following clinical trials [Reviewed in 147]. Thus, rIFN-β and anti-IFN-β antibodies in MS, especially NAbS, have been a focus in the medical community since the days of early therapeutic trials. There is a plethora of studies in this field, which has contributed to unravel its complexity, but there are still several areas that need further investigations.

Even though the occurrence of NAbS has been reported in clinical trials and in independent studies, the clinical importance of NAbS is claimed to be controversial [93, 96, 112, 115, 116]. One of the major problems of some of these studies is the relative short-term conclusion i.e. by 2 years. Generally, NAbS are detectable around 6-18 months after onset of treatment [109] and clinically important NAbS appear after 12-18 months of treatment, once the affinity maturation and the class switching of antibodies occurs [148]. Thus, clinical effects of NAbS are generally not apparent until the second year of treatment. Therefore, longitudinal studies have often underestimated the actual clinical impacts of NAbS on on-going IFN-β therapy. This has been implicated as one of the explanations for the claim that clinical impact of NAbS are controversial [141]. On the other hand, IFN-β is a partially effective drug and the clinical response also varies between patients. The clinical parameters that are used to monitor disease activity, progression and therapeutic responses such as relapse rates and EDSS are partly subjective measures and therefore tend to vary. Frequent monitoring by MRI is not feasible for every individual patient. Thus, practically, there is an aspect of difficulty for close and consistent monitoring of the effects of NAbS either clinically or by MRI imaging.

Our main methodological approach was to apply a cross sectional analysis on large samples of IFN-β treated MS patients to explore the aims mentioned in this thesis. The samples that were referred to our laboratory were analyzed retrospectively for a defined period of time. All patients had been treated for different lengths of time and had never been tested for NAbS before. This resembled a real-world situation and gave us a unique opportunity and an approach that most of the longitudinal studies lack, although these had the advantage of being prospective.

Approximately 32% of IFN-β treated MS patients were positive for NAbS and 16% of them had NAb titers high enough to reduce the bioactivity substantially, as evident from our first, second and third studies. These high titers tend to persist for a long time and hence it is likely that these patients have lost clinical efficacy of the drug for a foreseeable time. In the fourth study we evaluated the predictability of NAb status from the information of clinical activity of the patient. The detailed discussion of each study is given below:
5.1 STUDY I

Most of the previous studies have referred to the frequency of seropositivity as immunogenicity of IFN-β preparations. However, we found that immunogenic differences of IFN-β preparations can be described as both seropositivity and by the different titer levels of the antibodies induced. Therefore, we propose the terms seroprevalence for seropositivity and immunogenicity for titer levels.

Intramuscular IFN-β1a (Avonex) was found to be the least seroprevalent preparation compared to the subcutaneous high-dose IFN-β preparations. This observation was in line with the previous reports and thus reflects the validity of our method of NAb testing. The analysis on NAb titers indicated that Avonex-treated patients had the lowest immunogenicity in the whole range of NAb titers. As described in section 1.2.3.7, weekly low protein dose might be one of the factors determining the low seroprevalences and immunogenicity observed in Avonex. Betaferon-treated patients had the highest seroprevalence, but intermediate or low immunogenicity. Rebif22-treated patients had the highest immunogenicity with most of their positive patients having levels in the upper range of NAb titers, but with the group as a whole having intermediate seroprevalence. Thus, differences between preparations are more complex than mere seroprevalence. Therefore, we propose that both seroprevalence and immunogenicity have to be considered to assess the differences among IFN-β preparations in NAb inducing potential.

The analysis on treatment duration, to verify any confounding effects of time on seroprevalence, gave insignificant results. However, the proportion of patients with NAb tended to increase to a maximum at 4 to 5 years of treatment. Even though a significant proportion of (30%) patients were persistently NAb positive even after five years of treatment. This was surprising since the common notion is that NAb might disappear over time and thereby an increased occurrence of low seroprevalences towards longer treatment durations was expected. However, there might be a risk of under-representation of seroprevalences with time in longitudinal studies, due to the dropout of patients when NAb are persisting.

However, we observed slightly higher seroprevalence rates compared to the other studies. This might be due to the low assay cut off level (≥10 TRU/ml) in our MxA induction assay. Thus, even though high-dose IFN-β is generally associated with higher therapeutic potency, there is a parallel risk for increased NAb formation. The recently reported ‘Rebif new formulation’ (RNF) trial indicated that there is a significant reduction in seroprevalence by improving the formulation of Rebif while retaining its high potency [149].

The surprisingly high NAb seroprevalence of 13% (usually 6% in clinical trials) observed in patients on Avonex deserves a comment. This may indicate that the risk of NAb is really higher in “real world” treatment compared to the artificial setting of a trial. However, it is important to recognize that we lacked information regarding previous treatments. Thus, it is possible that some of the patients presently on Avonex had developed their NAb during treatment with a subcutaneous high dose IFN-β. In the case of comparisons between preparations, however, unrecognized switching in patients history will serve as a conservative factor, obscuring true differences rather than creating artifacts. Thus, the differences we observe are likely to be correct whereas we may have been unable to identify some other differences.
5.2 STUDY II

In the second study, the NAb titers were further explored to define functionally important titer levels in individual patients. To assess this, the in vivo response to IFN-β in patients with various titers of NAb was measured. As expected, we observed a strong negative correlation of NAb with the in vivo expression of both MxA and TRAIL mRNAs, in which, IFN-β bioactivity was reduced in a stepwise fashion with increasing NAb titer. We propose that this allows for a definition of cut-off titer levels for NAb titers that are useful in the clinical setting.

Thus, a NAb titer around 150 TRU/ml was found to be a critical demarcation in the whole spectrum of NAb titers ranging from 0 to >1280 TRU/ml. However, the level 150 TRU/ml is specific for our in-house protocol and individual laboratories may need to determine its own bioactivity levels of IFN-β following with a similar methodological approach.

It is clear that patients with NAb titers up to 150 TRU/ml usually have a well-retained MxA mRNA response, indicating that they have good in vivo therapeutic response to the drug. Patients with NAb titers between 150 TRU/ml and 600 TRU/ml have a low and partially retained expression of MxA, but still 3 times higher than in those with NAb titers above 600 TRU/ml. However, if this MxA response correlates with any significantly beneficial effect of IFN-β has to be investigated further, but considering the partial efficacy of IFN-β even in the absence of NAb, a reduction of some 80% of the bioactivity is likely to be detrimental. In the presence of NAb titers above 600 TRU/ml, 95% of the MxA-related bioactivity has been lost and the treatment in these patients can be assumed to be minimally efficacious.

A critical question is to what extent the in vivo response to MxA or IFN-β bioactivity can be translated into clinical or MRI-evident efficacy. A recent study that followed up 32 IFN-β treated MS patients who were categorized into 3 different NAb levels i.e. negative (0-20%), low (21-79%) and high (>80%) and IFN-β bioactivity was measured along with monthly MRI scans. They found correlations between titers of NAb, IFN-β bioactivity and MRI activity. However, MRI activity was variable and insensitive in the middle ranges of NAb or in the group with partial IFN-β bioactivity [136]. Therefore direct translation of IFN-β bioactivity needs further strenuous studies that closely monitor clinical and imaging parameters with NAb and bioactivity measurements, in order to firmly establish at what level NAb are interfering with clinical effect of the drug.

However, given the fact that MxA is a type-I IFN specific biomarker, the MxA up-regulation is assumed to reflect the expression of other interferon-stimulated genes (ISGs), which in turn presumably give rise to the pharmacological effects of IFN-β or the immunomodulatory effects of the drug in MS. Our own data, showing very similar findings for TRAIL mRNA, another IFN-β responsive transcript, support this notion. On the other hand, a high concentration of NAb blocks expression of typical interferon inducible genes, but the blocking effect on other IFN-β responsive genes are variable according to a small-scale micro array gene expression analysis by Hon and co-workers [150]. Thus, before applying direct relationship of neutralization to all receptor-mediated biological functions of IFN-β in MS, it is necessary to verify this in a large-scale gene expression analysis.
5.3 STUDY III

Having identified the functionally critical NAb titer cut off levels for bioactivity as assessed by the MxA assay, we focused on investigating NAb titer fluctuation or the dynamic nature of the NAb response in MS patients, especially across these functionally relevant bioactivity borders of NAb. This analysis is clinically important because future NAb status and stability of different NAb titer levels can be predicted and thereby, the requirement for repeated NAb tests and decisions on continuing of a treatment regimen can be evaluated.

The majority of NAb titers were stable and only 28% of all patients tested showed changes in their NAb titers during 1 to 3 year period. Especially those who were negative or had very high NAb titers remained stable. The persistent nature of high NAb titers has been reported before [137, 138] and is here supported further in a larger material. However, the middle range of NAb titers, especially around functionally critical level of 150 TRU/ml, tend to fluctuate suggesting that patients in this range should be followed-up with repeated sampling.

As expected, a decrease in NAb titers is overall more common than an increase, in these patients who have a mean treatment duration of close to three years. Thus, our results do not contradict previous observations of loss of NAbs over the course of many years [137, 138, 151], but show that this tendency is of minor importance in a time perspective relevant from a clinical point of view. For patients who have higher titers (>150 TRU/ml), there is only a 15% chance to regain good bioactivity in another 3 years time, although somewhat higher when closer to 150 TRU/ml. Conversely, when a patient is either NAb negative or shows titers below 150 TRU/ml, there is more than 90% chance that she or he will continue to have the therapeutic efficacy of the drug at least for another 3 years.

Somewhat surprisingly, in spite of considerable size of the sample, we failed to show a significant difference between preparations in a time horizon of 1-3 years and thus found little or no prognostic importance of choice of IFN-β preparation. A tendency of Betaferon NAbs to fluctuate somewhat more failed to reach significance compared to other preparations, although a previous study has claimed a greater chance for seroconversion in patients of this preparation [138]. Similarly, treatment duration and even interval between first and second sample failed to show influential effects on NAb titer fluctuation. Relatively short treatment duration i.e. median of 12 months may be a reason for insignificant results.

5.4 STUDY IV

One of the most critical aspects of NAbs is the influence on clinical efficacy of IFN-β treatment. All major controlled clinical trials and some independent prospective studies have shown the negative impact of NAbs on therapeutic efficacy of IFN-β at bioactivity, clinical and MRI response levels. However, different study designs, especially cross-sectional studies based on various populations and samples, sometimes give conflicting results and viewpoints.

One such recent report came out from the work of Goodin and co-workers that cross-examined a large sample of IFN-β1b treated MS patients collected from 3 different continents, namely North America, Europe and Australia [142]. Surprisingly, they reported a significantly lower seroprevalences in 3 samples (21%, 27% and 37% respectively) of patients who had an impression of worsening of the disease, and thereby claiming that NAbs are not important...
clinically. This observation led us to examine our data set in order to compare the different indications for NAb testing with the respective NAb statuses.

The compositions of the three samples of Goodin and co-workers were strikingly different; in the Australian group, the NAb testing was mandatory for all IFN-β treated MS patients and thus a population-based sample. However, there was no information in the referrals about the clinical state of the patients. In contrast, European and American samples were sent voluntarily by the clinicians in response to free NAb tests offered from manufacturer of IFN-β1b. However, the authors have pointed out that the worst aspect of their data set was the ascertainment of these clinical statuses; North American cohort might have been over-represented by the indication ‘disease worsening’ by way of clinician’s ‘forced choice’ to get a NAb test done for free even in patients with good clinical response for IFN-β. In addition, there are significant differences in treatment durations between the three cohorts. The North American and European samples consisted of more progressive MS patients while the Australian sample was exclusively early RRMS. Thus, enrichment of patients with worsening of disease tended to be higher in European and North American samples despite the actual influence of NABs.

Our study compared NAb statuses in both ‘worsening’ and ‘routine’ samples that were obtained from a population in which biases and enrichments have been introduced randomly and equally compared to samples of Goodin and co-worker’s study. We found comparable seroprevalences in the routine (31%) versus the worsening (30%) groups. These figures are in line with the rates of our previous known figures of seroprevalences. However, we also failed to demonstrate any increased incidence of NABs in patients who are experiencing a worsening of the disease.

The important query is why we fail to demonstrate an association between clinical worsening and NAb positivity. IFN-β is a partially efficacious drug that causes about 30% of reduction in relapse rates. Thus at least 70% of all relapses, at the group level, will occur anyhow, each time potentially indicating loss of efficacy. In addition, patients may progress in the absence of a relapse, such as in early secondary progression, a kind of progression that IFN-β is unlikely to prevent. For example, unpublished data from the Swedish MS registry show that approximately 25% of IFN-β treated MS patients have SPMS. A similar study has reported that 21% of all IFN-β treated patients were in fact SPMS [16]. In the Goodin’s study, both North American and European samples included nearly 13-14% of SPMS.

There were some biases introduced in our study; patients treated with Betaferon were more often selected for NAb testing because of a worsening of disease compared to patients treated with Avonex and Rebif, probably because they were older and had longer durations of treatment than patients treated with the other two preparations. Patients treated with Avonex were on the other hand more often referred for routine testing. There is some controversy in this observation. One would expect less frequent routine testing in Avonex treated patients, because it is known that the drug rarely induces NABs. On the other hand, the Avonex treated patients were younger than patients on Betaferon and had shorter duration of the disease than Betaferon and Rebif treated patients, thus more likely to be stable, and sampled routinely. In addition, since there is some evidence of better efficacy of higher dosage and high frequency IFN-β treatment, Avonex may be more often prescribed to patients with less active MS [152, 153]. Thus, in a non-randomized cohort of patients, it is clear that there are biases making comparisons of preparations scientifically questionable.
Another limitation of our study is that data collection from clinically used referral forms is less precise than data collection from e.g. clinical trials. Thus, an increased ‘noise’ level may serve to diminish an existing difference such as an increased NAb frequency in worsening patients. On the other hand, many aspects of clinical trials are different from the clinical reality in which drugs are prescribed, emphasizing the importance of ‘real world data’ [154].

NAb positive patients were more often sampled due to ‘other indications’. It is difficult to draw conclusions from these data since the number of patients with this indication was small as well as heterogeneous, for example including reasons likely to be acting in opposite directions, such as absence and presence of IFN-β side effects.

5.5 METHODOLOGICAL CONSIDERATIONS

The MxA induction assay is a bioassay involving several steps such as in vitro culturing and stimulation of a cell line. The outcome response measures in these biological systems are inherently variable. Therefore, one of the typical, but unavoidable methodological, issues is the assay variability. In a pilot study we determined the interassay variability for our lab, to be 1-25% (mean 5%). Thus, assay variability can alter the sensitivity of the NAb test and can cause variation of NAb results. Of all four studies, assay variation is mostly critical during interpretation of NAb titer fluctuation. But this risk of assay variability is applicable for all samples in general in an equal manner. Therefore it is less likely that assay variation should be grossly affecting the overall pattern of titer fluctuation.

In addition, we have attempted to validate our NAb assay with other laboratories and found a good agreement in the repeatability of results with minimum inter-laboratory variation. To compare the MxA titer of 150 TRU/ml with CPE titer, a pilot study of 15 serum samples were crosschecked with CPE assay. Titers in MxA are 4-5 times higher than CPE-titer. Thus MxA 150 TRU/ml in our assay may correlate with about 20-30 neutralizing units in the CPE. A similar correlation was done by McKay and co-workers [155], who studied both assays in parallel, and who reported that a CPE titer of 20 was equivalent to an MxA–titer of 180. These differences are primarily due to the different methodological principles in end point reading of the neutralizing assay component i.e. viral cytopathic effect readout and MxA protein measurement by ELISA.

The individual variation in MxA mRNA levels was also observed in the study two. This could be explained by differences between individuals in their IFN-β responsiveness due to for instance, differences in compliance, injection techniques and other practical reasons. Of these, sampling time was most critical for in vivo MxA mRNA due to the kinetics of MxA mRNA expression. The optimal sampling time was 12 hours after the last IFN-β injection to observe a peak MxA mRNA expression [125, 146]. In this study, the majority of the patients came to the clinic for blood sampling 12 hours after their last injection, whereas a few came somewhat later, which could be seen as a clear drop in MxA expression. Even though MxA mRNA expression has superior specificity levels over protein measurement, the narrow sampling window raise the question of feasibility of the method.

One could argue that the MxA induction (or CPE) NAb tests might be less relevant if it cannot be directly translated into bioactivity, as discussed above. However, measurements of IFN-β in vivo bioactivity with MxA mRNA or protein alone, as an alternative to NAb testing [125, 131, 134],
may also encounter shortcomings such as: inability to distinguish between reduced drug efficacy due to NAbs and therapeutic failure due to other factors such as poor compliance; logistical difficulty in sampling patients at a narrow time window for optimal MxA mRNA expression compared to determining a constantly present level of serum NAbs; and the loss of information on titer level which is important.

5.6 CONCLUSION

In conclusion, there are differences in seroprevalence and immunogenicity of IFN-β preparations that are commercially available to treat MS patients. Development of NAbs reduces bioactivity of IFN-β and NAb titer around 150 TRU/ml marks a functionally critical cut off level. Patients with titers below this level showed retained IFN-β bioactivity while patients with titers above 600 TRU/ml was clearly associated with loss of IFN-β bioactivity. Comparing titer fluctuation across these borders revealed that NAb titers are relatively stable over 1-3 years, especially those which are negative or very high, whereas titers close to functionally critical levels have a chance of changing to titers that correspond to losing or regaining IFN-β bioactivity. Therefore information on titer levels is useful in the management of IFN-β treated MS patients who have developed NAbs. However, assessment of the predictability of NAb status on disease activity or vice versa, failed to show an association. In other terms, we did not observe any increased NAb positivity among the referrals with impression of disease worsening.

Development of antibodies to rIFN-β is possibly due to the breach of immunological tolerance to IFN-β. However the exact mechanism has not been delineated yet. We could describe the occurrence of this process in relation to a population-based sample of MS patients as seroprevalence and its severity as immunogenicity. The dynamic nature of this response was evident in the observed patterns of titer fluctuation. However, the most intriguing aspect of NAbs is the interactions with other pharmaco-biological processes like bioactivity and therapeutic effects of IFN-β in MS (figure 9). The bioactivity of IFN-β in MS patients is measurable and we could demonstrate the effects of NAbs on IFN-β bioactivity even at individual patient’s levels. However, by this means, still we can’t directly translate the relationship between NAbs and bioactivity to yet unclear overall immunomodulatory functions or therapeutic effects of IFN-β in MS. Thus, the clinical impact of NAbs, which is the most critical aspect of all, is indeed a complex phenomenon and is essentially an overall outcome of the interactions of above processes. This might be one reason for why differential results that have been observed in studies attempting to correlate NAb status with clinical worsening of the disease or vice versa. On the other hand, differences in temporal relationships of these processes, clinico-pathological heterogeneity and differences in IFN-β therapeutic responses among MS patients make the whole scenario even more challenging for the clinician and for the researcher.
Figure 9: Schematic representation of interactions between NAbs and IFN-β in MS
5.7 FUTURE PERSPECTIVES

- Correlation of clinical or MRI surrogate markers of disease activity or disease progression with IFN-β bioactivity is a step further to the observations made in study two. This would address one of the major controversies regarding NAbs, that is, the clinical importance of NAbs. Identification of specific clinical or MRI surrogate markers for disease activity and progression might be a first step during this exercise. Follow up of patients with repeated NAb testing, close MRI and clinical assessment is a requirement while enrolling a sufficient number of patients to have enough power to get rid of the variability outcome measures.

- The analysis of NAb titer fluctuation was restricted to a sampling interval of 3 years. This can be further analyzed as more samples are collected in coming years. This would enhance the predictability of future NAb status and stability for a longer duration.

- IFN-β is also important in innate immune response against viral infections. NAbs can theoretically neutralize endogenous IFN-β even though there are no such reports yet. When the ongoing IFN-β therapy is stopped, patients might face this situation, especially when there are sufficiently high NAb titers in the blood. We hypothesized that these patients might experience increased susceptibility to viral infections, which may be clinical or sub-clinical in nature. So far there is no evidence in this regards. Therefore we are focusing on this aspect and studies are underway to investigate following aims:
  - To demonstrate in vitro effects of NAbs on endogenous IFN-β.
  - To assess the persistence or disappearance of NAbs after cessation of IFN-β treatment in patients and to follow them up to assess a hypothetically altered incidence of viral infections.
  - To test this hypothesis in a mouse model with NAbs by challenge with viral infection to assess any increased susceptibility for infections.

- One of the fundamental questions of anti-IFN-β NAbs is the mechanism of development and persistence of NAbs, like the dependence of T cells and the survival of plasma cells. Moreover, only a sub-group of patients develops NAbs and it is unknown what is special with these patients compared to those that apparently never develop NAbs. Currently it has been hypothesized that NAbs develop due to the breakdown of immunological tolerance to IFN-β. How this occurs is not clear, but apparently the immunological tolerance against IFN-β is not complete. Similarly, why NAbs disappear after a while in some patients and not in others also needs evidence-based explanation.

- As it is mentioned in the main conclusion, the net outcome of neutralization of IFN-β and its clinical manifestation is complex, partly due to the ill-defined immunomodulatory functions of IFN-β in MS. Large-scale gene expression studies are necessary, especially in the presence of NAbs in IFN-β treated MS patients. This might help to further identify better surrogate markers for bioactivity measurement and perhaps markers that are in direct relation to MS pathogenesis.
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7 REFERENCES


8 APPENDICES

The referral form

**Provtagning för analys av neutraliserande antikroppar mot β-Interferon**

**Serum**
- Prov tas minst 48 tim efter injektion eller direkt före nästa
- 10 ml blod tages som en venopunktion i senumväv
- Skall centrifugeras inom 1 timme vid 3000 rpm i 10 min
- Efter centrifugering skall minst 1-2 ml föras över i steril polypropylen rör och frysas i −20°C
- Förvaras i −20°C i väntan på transport.

**Transport**
- Provet måste sändas fryst med tons (se till att frigörlådan är fyld med is)
- Laboratoriet tar endast emot prover som skickats må- torsdag och aldrig kommer till laboratoriet under kontorstid och ej i anslutning till helg.
- Före transport skall e-postmeddelande skickas till anna.ljungberg@neurotec.li.se och skall innehålla följande info:*
  - Datum för beriktnad transport av prov
  - Adress och telefonnummer till den som skall ta emot provet
  - E-post och ID
  - Namn, adress och telefonnummer till den som skall ta emot provet
  - Information om var provet skall skickas

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*Provet skall skickas till: Läkarlab, Neurologiska kliniken R52, Huddinge Universitetssjukhus, 141 86 Stockholm*