ANTI-DRUG ANTIBODIES IN PATIENTS WITH MULTIPLE SCLEROSIS

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory disease affecting the brain and spinal cord and it is the main cause of neurological disability among young adults. Recombinant interferon beta (IFNβ) and natalizumab are commonly used disease-modifying drugs that reduce disease severity. Even though these treatments show beneficial clinical effects they are associated with the development of anti-drug antibodies (ADAs), which at high titer levels reduce drug efficacy. Although ADAs are known to adversely affect the clinical effect of the treatment on a group level, the treatment response in individual patients is less characterized. In addition, it is unknown why only a subgroup of treated MS patients develops ADAs. The objective of this thesis was to identify biologically relevant ADA titer cut-points that can be used to predict treatment response and persistence of ADAs in individual patients, and to investigate if genetic and immunological factors influence the development of ADAs in MS patients.

MS patients analyzed for the presence of ADAs against IFNβ or natalizumab in the routine NAb laboratory at Karolinska Institutet were included in this project. In Sweden, NAb monitoring became clinical practice in 2003 and during 2003-2004 the overall seroprevalence of neutralizing antibodies (NAbs) against IFNβ was 32%. When the NAb seroprevalence was analyzed five years later, in 2009-2010, the overall frequency of NAb-positive patients had decreased significantly to 19%. Importantly, the greatest reduction was observed in patients with high NAb titers (study I). By correlating the in vivo IFNβ bioactivity with patients’ NAb titers we identified that a NAb titer of 150 TRU/ml is a biologically functional cut-point for treatment response, since titers above 150 TRU/ml completely block IFNβ bioactivity (study II). Furthermore, characterization of ADA responses in natalizumab-treated patients revealed that the level of total anti-natalizumab antibodies in a first positive sample can be used to predict patients at risk of becoming persistently antibody positive (study V).

It is known that factors such as protein modifications and/or impurities impact the immunogenicity of IFNβ, which can explain the variation in NAb positivity between IFNβ preparations. In addition, since only a subgroup of IFNβ-treated patients develops NAbs, patient-related factors are likely to influence the immunogenicity of IFNβ. In study III, we hypothesized that MS patients with and without intrathecal production of oligoclonal IgG bands (OCB) have different propensities to induce humoral immune responses. The presence of OCB was found to be associated with NAb development, and this risk was confined to NAbs against IFNβ-1a. From these results we proposed that MS patients with and without OCB differ immunologically, potentially influenced by distinct human leukocyte antigen (HLA) alleles. The role of HLA in the immunogenicity of IFNβ was further investigated in study IV, in which we found that HLA-DRB1*15 carriage was associated with increased risk of developing NAbs. Stratification on type of IFNβ preparation showed that HLA-DRB1*15 increased the risk of NAbs against IFNβ-1a, while HLA-DRB1*04 increased the risk of NAbs against IFNβ-1b, indicating that there is an IFNβ preparation-specific genetically determined risk to develop NAbs.

Overall, these results can be used to assist when making decisions about whether treatment should be discontinued or not. In addition, the identification of factors contributing to the immunogenicity of protein therapeutics can increase our understanding of the immunological mechanisms leading to ADA responses, possibly resulting in less immunogenic drugs in the future.

II. Anti-interferon beta antibody titers strongly correlate between two bioassays and *in vivo* biomarker expression, and indicate that a titer greater than 150 TRU/ml is a biologically functional cut-point. Hermanrud C, Lundkvist Ryner M, Engdahl E, Fogdell-Hahn A. Accepted for publication in *J Interferon Cytokine Res*. 2013 Oct

III. Multiple sclerosis patients lacking oligoclonal bands in the cerebrospinal fluid are less likely to develop neutralizing antibodies against interferon beta. Lundkvist M, Greiner E, Hillert J, Fogdell-Hahn A. *Multiple Sclerosis Journal*. 2010 Jul;16(7):796-800.


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<td>Anti-drug antibodies</td>
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<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>AR</td>
<td>Absolute risk</td>
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<tr>
<td>BAb</td>
<td>Binding antibody</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>BCR</td>
<td>B-cell receptor</td>
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<tr>
<td>BP</td>
<td>Biopharmaceutical</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CIS</td>
<td>Clinically isolated syndrome</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CXCL-10</td>
<td>C-X-C motif chemokine-10</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EC₅₀</td>
<td>Effective concentration₅₀</td>
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<tr>
<td>EIMS</td>
<td>Epidemiological Investigation of Multiple Sclerosis</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>GEMS</td>
<td>Genes and Environment in Multiple Sclerosis</td>
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<tr>
<td>H₂SO₄</td>
<td>Sulfuric acid</td>
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<tr>
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<td>Human herpes virus-6</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HPRT1</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
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<td>HSA</td>
<td>Human serum albumin</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IFNAR</td>
<td>Interferon-alpha receptor</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Interleukin</td>
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<td>IMSE</td>
<td>Immunomodulatory Multiple Sclerosis study</td>
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<td>IQR</td>
<td>Interquartile range</td>
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<td>ISG</td>
<td>Interferon-stimulated genes</td>
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<td>ISGF</td>
<td>Interferon-stimulated gene factor</td>
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<td>ISRE</td>
<td>Interferon-stimulated response elements</td>
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<td>ITI</td>
<td>Immune tolerance induction</td>
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<tr>
<td>IU</td>
<td>International unit</td>
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<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
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<td>MGA</td>
<td>Myxovirus resistance protein A gene expression assay</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>MxA</td>
<td>Myxovirus resistance protein A</td>
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<tr>
<td>MxB</td>
<td>Myxovirus resistance protein B</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NAb</td>
<td>Neutralizing antibody</td>
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<tr>
<td>OCB</td>
<td>Oligoclonal IgG band</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PPMS</td>
<td>Primary progressive multiple sclerosis</td>
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<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>RLU</td>
<td>Relative luminescence unit</td>
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<td>RNF</td>
<td>Rebif New Formulation</td>
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<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis</td>
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<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SMS registry</td>
<td>Swedish Multiple Sclerosis registry</td>
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<td>SPMS</td>
<td>Secondary progressive multiple sclerosis</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>STOP MS</td>
<td>Stockholm Prospective Assessment of Multiple Sclerosis</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinase</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRU/ml</td>
<td>Tenfold reduction units per milliliter</td>
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<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VLA</td>
<td>Very late antigen</td>
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<td>VZV</td>
<td>Varicella zoster virus</td>
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1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that affects millions of people worldwide and it is the main cause of neurological disability among young adults [1]. In Sweden, the disease affects 189 per 100,000 individuals, and women are more than twice as often affected [2].

The disease is characterized by development of inflammatory demyelinating white matter lesions, plaques, in the brain and spinal cord. In lesions where myelin and the myelin-producing oligodendrocytes are permanently lost, astrocytes form glial scars (sclerae), and as the name multiple sclerosis implies, these scars form at multiple sites in the CNS. Demyelination continues in both white and grey matter, and over time both axons and neurons are injured and lost. In affected patients the chronic inflammation and neurodegeneration will in time lead to gradual accumulation of disability [3], which impacts on the patients’ health-related quality of life [1]. Although MS is usually not the direct cause of death, patients have a slightly reduced life expectancy compared to the general healthy population [4].

Disease symptoms usually start between the ages of 20 and 40, but subclinical disease activity possibly begins before clinical presentation. Depending on where the lesions occur in the CNS, patients may suffer loss of neurological functions in motor, sensory, visual and autonomic systems. More specific signs and symptoms that frequently occur at disease onset are, for example, weakness in arms and legs, impaired movement, dizziness, inflammation of the optic nerve, and double or loss of vision [3].

Depending on the clinical picture and disease course, MS is classified into relapsing-remitting (RR), primary progressive (PP) and secondary progressive (SP) disease. About 80-90% of patients receive the diagnosis relapsing-remitting disease, which is characterized by acute attacks of disease symptoms (relapses) followed by periods of full or partial recovery (remission) from symptoms. In 80-90% of the patients with RRMS, the frequency of inflammatory driven relapses decrease over time, and within 10 to 20 years from onset the disease evolves into a secondary-progressive phase with gradual accumulation of disability. Primary-progressive disease affects only 10-15% of patients with MS, and is characterized by less pronounced inflammation and with accumulation of disability from disease onset [3,5,6].

For a diagnosis of MS to be made, according to the McDonald criteria, there needs to be evidence of at least two demyelinating events that are separated in time and space. This is done primarily by clinical history and neurological examination, but paraclinical investigations can be made to support the diagnosis or disease course. These tests include examination of lesion burden and axonal loss using magnetic resonance imaging (MRI), and detection of intrathecal synthesis of oligoclonal immunoglobulin (Ig) G by cerebrospinal fluid (CSF) analysis [7,8]. The presence of oligoclonal IgG bands (OCBs) or elevated IgG levels in CSF compared to paired serum or plasma samples is a hallmark of MS, and is detected in 88% to 100% of patients (reviewed in [9]).
1.1.1 Pathogenesis and disease mechanisms

The disease is believed to be initiated when CD4+ T cells, by unknown mechanisms, are activated in the periphery and enter the inflamed CNS by transmigration across the blood-brain barrier (BBB). Within the CNS, the activated T cells recognize their specific target antigens (e.g. myelin-derived peptides) presented by antigen-presenting cells (APCs). This leads to increased production of pro-inflammatory factors, e.g. cytokines and chemokines, by activated CD4+ effector T cells, infiltrating macrophages, and CNS resident microglia and astrocytes, which attract additional immune cell subsets (cytotoxic CD8+ T cells, B cells and innate immune cells) into the CNS (reviewed in [10]). The toxic environment that is produced by soluble inflammatory mediators, together with targeted myelin destruction and killing of myelin-producing oligodendrocytes by CD8+ effector T cells and antibody-mediated mechanisms, result in focal demyelination and axonal loss (reviewed in [11,12]).

T cells (CD4+ and CD8+) and macrophages (antigen-presenting cells, APCs) are the most abundant immune cells present in MS lesions, while B cells and antibody-producing plasma cells are found at lower frequencies [13]. In addition, studies on animals with experimental autoimmune encephalomyelitis (EAE), a model for MS, have shown that myelin-reactive CD4+ T cells are necessary for transfer of disease from affected to non-affected animals [14]. These findings have led to the assumption that MS is a T-cell mediated autoimmune disease.

B cells and antibodies were previously not considered to play key roles in the disease process. Today it is evident that both components are involved in mechanisms contributing to disease. The detection of clonal expansion and somatic mutation of B cells in the CNS and CSF [15-17], which participate in the intrathecal synthesis of IgG and OCBs [18-20], indicates that these responses are T cell-dependent and antigen driven. In addition, clonal B-cell expansion is detected in a majority of patients with a first clinical episode suggestive of MS, or clinically isolated syndrome (CIS). These clonal B cells seems to develop before detectable OCBs and MRI lesions, suggesting that antigen-specific B-cells are involved in the early phase of disease [16]. Furthermore, treatment of MS patients with a B-cell depleting monoclonal anti-CD20 antibody, rituximab, rapidly reduced inflammatory lesions on MRI [21], suggesting that B cells also are involved in formation of acute lesions.

Although B-cell mediated humoral immunity is important for disease, this does not seem to be the case in all patients with MS. The fact that intrathecal OCBs, a typical finding in MS, are undetectable in 5% to 10% of patients with MS indicates that this group differ immunologically from patients with OCBs. Indeed, these patients make up two immunogenetically different subgroups of MS as they have been found associated with distinct human leukocyte antigen (HLA) class II alleles, HLA-DRB1*15:01 with OCB-positive MS and HLA-DRB1*04:04/04:05 with OCB-negative MS [22-24]. Several studies have also shown that OCB-negative MS is associated with better clinical outcome in terms of lower relapse rate, less disability and slower disease progression [22,25-28], whereas others have found no difference [23,29,30] or even opposite results [31]. Findings also suggest that patients with OCB-negative MS respond better to interferon beta therapy than those with OCB-positive MS [32]. Altogether, this might indicate that these groups differ in respect to humoral immunity and possibly also in disease mechanism.
1.1.2 Factors influencing disease

1.1.2.1 Genetic factors

Although the etiology of MS is unknown, both genetic and environmental factors have been identified to influence susceptibility to disease. Among the genetic factors identified so far, the most important risk factors are located to the HLA region. The strongest associated gene, the HLA class II allele HLA-DRB1*15:01 [33], has been reported to increase the risk for MS about 3-fold, whereas a protective effect has been shown for the HLA class I allele, HLA-A*02 [33-35]. Given that HLA plays a central role in antigen presentation to T cells, and that most other associated genes also seems to be important for the immune system [33,36], it is likely that both antigen presentation and immune dysregulation are important mechanisms in the development of MS. However, the genetic susceptibility can only partially explain why some individuals become affected, since the concordance rate in monozygotic twins has been reported to be around 24-26% while it is around 2-3% for dizygotic twins and non-twin siblings [37,38]. This indicates that additional environmental factors are needed to trigger the disease in genetically susceptible individuals.

1.1.2.2 Environmental and life-style factors

1.1.2.2.1 Virus infections

Based on results from both epidemiological studies and studies on other demyelinating CNS diseases, virus infections have been extensively investigated as possible triggers of MS (reviewed in [39]). Two candidate viruses suggested to play a role in MS are Epstein-Barr virus (EBV) and human herpes virus (HHV)-6. Epidemiological studies have reported an increased risk for MS in EBV-infected individuals, especially if primary infection occur in adulthood and results in infectious mononucleosis [40-42]. For HHV-6, serological studies have reported higher prevalence of anti-HHV-6 IgM and IgG antibodies in MS patients compared to controls [43,44]. Studies have also reported that viral DNA or viral products from both EBV [45,46] and HHV-6 [47,48] are present in brain and active lesions of MS patients, but not in controls. Furthermore, immunological studies on CSF in MS patients have shown that fractions of OCB are reactive against specific antigens from both EBV and HHV-6 [49-51]. Although other studies have reported contradictory results, viruses may still play a role in the disease.

1.1.2.2.2 Sunlight exposure and vitamin D

Observations of higher MS prevalence in areas closer to the north and south poles suggest that low sunlight exposure and/or low vitamin D levels increase disease susceptibility [52]. This is supported by the fact that both factors are recognized to have immune-modulating potentials [53], possibly affecting the inflammatory response in MS.

1.1.2.2.3 Smoking

Several epidemiological studies have shown that tobacco smoking significantly increases the risk of MS [54-56]. The exact mechanisms behind this increased risk in smokers are not known, but some substances in tobacco smoke have modulatory effects on immune cell function and can impair both cell-mediated and humoral immunity [57,58], and it is possible that such alterations influence the inflammatory response in MS.
1.2 TREATMENTS IN MULTIPLE SCLEROSIS

Since the cause of MS is unknown there is no cure for the disease. However, there are a number of disease-modifying treatments that reduce disease symptoms as well as disease severity and disability accumulation over time. In patients with severe and rapidly progressing MS autologous hematopoietic stem cell transplantation therapy is now being used. Although this therapy has shown promising results in MS patients it is also associated with an increased risk of mortality [59,60]. Thus, transplantation therapy is carried out only in patients where other therapies have failed. For the large group of MS patients with a relatively better disease course, different types of immunomodulatory treatments are used instead, which have shown to improve the disease to some extent, or even give very good results. Two of the immunomodulatory treatments most commonly used today are interferon beta and natalizumab.

1.2.1 Interferon beta treatment

1.2.1.1 Type I interferons

Interferon beta (IFNβ) is a cytokine that belongs to the IFN family, which includes three groups of related cytokines: type I IFNs, type II IFN and the recently described type III IFNs. In humans, the type I IFN group includes IFNα, IFNβ, IFNε, IFNκ and IFNω. In comparison, IFNγ is the only type II IFN and the type III IFN group consists of three IFNλ molecules. These types of IFNs bind to distinct receptor complexes and thereby they have different biological functions than the cytokines belonging to type I IFNs (reviewed in [61]).

Type I IFNs are widely expressed in our bodies. These cytokines are a part of the innate immune response and are important in the first-line defense against viral infections by directly inhibiting viral replication in infected cells [62]. Of the type I IFNs, IFNα and IFNβ are the most studied. In response to viruses and viral components, e.g. double stranded RNA, high levels of IFNα and IFNβ are produced by plasmacytoid dendritic cells (DCs) and fibroblasts respectively (reviewed in [61]). However, all nucleated cells can produce type I IFNs if they become infected with virus.

The type I IFNs share sequence similarities and they all signal through the same type I IFN receptor, consisting of the two subunits IFNAR1 and IFNAR2. The IFNAR1/2 complex is expressed on the surface of most cell types, which account for their important role in innate immunity. Although all type I IFNs signal through the same receptor complex they interact with the receptor differently, which account for their specific biological functions (reviewed in [61,63]).

The intracellular part of each receptor subunit interacts with a specific molecule of the Janus activated kinase (JAK) family: IFNAR1 with the JAK tyrosine kinase 2 (TYK2) and IFNAR2 with JAK1. When type I IFNs, e.g. IFNβ, bind to the extracellular domain of the receptor the IFNAR1 and IFNAR2 subunits dimerize, which results in autophosphorylation and activation of TYK2 and JAK1. The activation of JAKs result in subsequent phosphorylation and dimerization of STAT1 and STAT2 (signal transducer and activator of transcription 1/2); this STAT dimer then associates with IRF9 (IFN-regulatory factor 9) which leads to the formation of the ISGF3 (IFN-stimulated gene factor 3) complex. The ISGF3-trimer complex is then able to translocate to the cell nucleus where it binds to specific promoter elements in the DNA,
so called ISREs (IFN-stimulated response elements), and start the transcription of interferon-stimulated genes (ISGs) (reviewed in [61,63]).

Type I IFN signaling alters the expression of hundreds of genes which encode for products that for example have antiviral (myxovirus resistance proteins; MxA and MxB), anti-proliferative/pro-apoptotic and immunomodulatory effects. These biological activities of the type I IFNs has made them useful as treatments for several disorders. Today, IFNα is used for treatment of different cancers and chronic viral infections, whereas IFNβ is used as a treatment in MS (reviewed in [61]).

1.2.1.2 Recombinant interferon beta

1.2.1.2.1 Product properties

Two types of recombinant IFNβ products are used for treatment of MS, IFNβ-1a and IFNβ-1b. The IFNβ-1a preparations are injected either at 30 µg intramuscularly (i.m.) once a week or at 22 µg and 44 µg subcutaneously (s.c.) three times a week, and the IFNβ-1b preparation is injected at 250 µg s.c. every other day. Recombinant IFNβ-1a is expressed in a Chinese hamster ovarian (CHO) cell line and is identical to human IFNβ, both in primary sequence (166 amino acids) and in structure. Recombinant IFNβ-1b is expressed in a prokaryotic system using Escherichia coli, and therefore it lacks the N-terminal methionine (Met1) and the asparagine-linked glycosylation at position 80 (Asn80). To improve the quality of the protein, IFNβ-1b also carries a cysteine-to-serine substitution at position 17 (Cys17Ser). Thus, IFNβ-1b differs both in amino acid sequence (165 amino acids) and in carbohydrate content compared to human IFNβ and recombinant IFNβ-1a. Despite these structural differences both recombinant IFNβ products have similar biological activities [64]. However, when comparing these two products in bioactivity assays, the IFNβ-1a has been shown to be more potent than IFNβ-1b [65]. The lower potency of IFNβ-1b is likely to be a result of the lack of glycosylation, since this post-translational modification is considered to stabilize the protein structure. The lack of glycosylation also makes IFNβ-1b less hydrophilic in solution, making it more prone to aggregate compared to IFNβ-1a. As a result of the lower potency of IFNβ-1b, it is given at a higher weekly dose compared to the IFNβ-1a preparations. The weekly dose of each preparation is: 30 µg for i.m. IFNβ-1a, 66 µg or 132 µg for s.c. IFNβ-1a, and 875 µg for IFNβ-1b.

1.2.1.2.2 Therapeutic efficacy

Since IFNβ is a pleiotropic cytokine with a complex mechanism of action the exact mechanism(s) behind its beneficial effect in the treatment of MS is still not completely elucidated. Although viral infections might play a role in MS and IFNβ has potent antiviral functions it is the immunomodulatory properties of IFNβ that are considered to be the most relevant for its therapeutic effect in MS. IFNβ has both pro- and anti-inflammatory properties. In diseases like systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and neromyelitis optica the pro-inflammatory effects of IFNβ has been shown to augment the autoimmune inflammatory responses. In contrast, IFNβ seems to exert anti-inflammatory effects in MS (reviewed in [66,67]).

In MS, IFNβ can prevent breakdown of the BBB, which is an early event in the disease process that promotes lesion formation, by reducing expression of proteolytic enzymes like matrix metalloproteinase-9 (MMP-9) and increasing expression of the MMP-9 inhibitor TIMP-1 (tissue inhibitors of metalloproteinase-1) [68-70]. IFNβ treatment has also been reported to down-regulate cell surface expression of the α-4 integrin,
part of the very late antigen-4 (VLA-4), on T cells (CD4+ and CD8+) [71,72], and to increase the expression of soluble vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells [73]. Thus, the strengthened integrity of the BBB and the reduced capacity of T cells to cross the BBB may be two of several mechanisms by which IFNβ induces its therapeutic effect in MS.

IFNβ also seems to shift the cytokine profile from a pro-inflammatory to an anti-inflammatory state, by reducing the production of IL-12 and IFNγ while increasing IL-10 production [74-76]. In addition, recent findings suggest that IFNβ suppress the differentiation of IL-17-producing T helper 17 cells, which are important in autoimmune responses [77], and this effect seems to be mediated by IFNβ-induced production of IL-27 [78]. Furthermore, IFNβ has been suggested to have neuroprotective effects by promoting astrocytes to produce nerve growth factor or by neuronal protection [79,80].

1.2.1.2.3 Responders and non-responders to interferon beta treatment

Although IFNβ significantly reduce disease activity and severity, it is only partially effective and around 20% of treated patients show no or poor response to IFNβ. Pharmacogenomic studies have identified genetic variations, i.e. single nucleotide polymorphisms (SNPs), associated with genes for IFNAR2, ion channels and signal transduction pathways that differed between responders and non-responders to treatment [81,82]. Results from genome-wide expression studies also suggest that differential expression of IFNβ-inducible genes is correlated with response to IFNβ treatment [83,84].

1.2.2 Natalizumab

Natalizumab is approved for the treatment of RRMS and has proven to be very efficient to reduce relapse rates, lesion formation and risk of disability progression, both in clinical trials [85,86] and in post-marketing settings [87-89]. Today, in Sweden, natalizumab is used as a second line treatment in RRMS patients who do not tolerate or respond well to first line treatments such as IFNβ, but it can also be used as a first line treatment in MS patients with an aggressive disease onset.

Natalizumab is a recombinant monoclonal antibody that binds to the α4 subunit of the α4β1 integrin (VLA-4) and the α4β7 integrin (reviewed in [90]). Based on the results from studies in the EAE model, it was shown that antibodies which blocked the α4β1 integrin inhibited the binding of T cells and monocytes to the inflamed BBB. Furthermore, antibodies against α4-integrin prevented the development of EAE by reducing infiltration of immune cells in the CNS [91]. The main ligand for VLA-4 is the vascular cell adhesion molecule (VCAM)-1, which is upregulated on endothelial cells during inflammation. Natalizumab acts by blocking this interaction which prevents T cells from crossing the BBB to enter the CNS, reducing inflammatory response as well as subsequent reduction in myelin and axonal damage (reviewed in [92]).

Natalizumab was originally a mouse monoclonal antibody, but it was later humanized by grafting the complementarity-determining regions of the murine antibody onto a human IgG4 antibody backbone [93]. Natalizumab has been found to be generally well tolerated [85-87], but despite its similarity with a human IgG4 antibody treatment with natalizumab has been shown to induce the development of ADAs that can reduce the
therapeutic efficacy [85,86]. Thus, patients who develop persistent ADA responses should discontinue treatment.

1.3 ANTI-DRUG ANTIBODY DEVELOPMENT

Both IFNβ and natalizumab are examples of the increasing numbers of biological protein therapeutics that have been developed to treat disease, also known as biopharmaceuticals (BPs). Since BPs are produced to be similar or almost identical to the human homologous protein, these drugs are expected to be well tolerated by the immune system. Despite these similarities, BPs can still be considered as foreign by the immune system, subsequently leading to breakage of tolerance and development of anti-drug antibodies (ADAs). Thus, development of ADAs is an unwanted immunological response to BPs and it is associated with a decrease or complete loss of therapeutic efficacy. In addition, ADAs can cross-react with the endogenous human protein, which at worst can result in severe or life-threatening side effects. An example was the development of ADAs against recombinant erythropoietin that caused pure cell aplasia in patients treated for chronic renal failure, since the ADAs neutralized the effect of the endogenous protein as well [94].

1.3.1 Immunological mechanisms behind antibody development

Antibodies against BPs can be divided into two subtypes: binding antibodies (BAbs) and neutralizing antibodies (NAbs). BAbs are considered to have little or no effect on the pharmacological response to BPs, whereas NAbs interfere with the bioactivity of BPs and reduce the treatment effect. NAbs are typically of the IgG subclass, indicating that humoral immune responses to BPs are mainly induced by activation of B cells with help from T cells (CD4+ T helper cells), i.e. classical immune response.

In a T-cell dependent B-cell response, the antigen (e.g. BP) is taken up and processed by professional APCs such as DCs. Processed antigen peptides are presented on the cell surface by HLA class II molecules to antigen-specific T cells that then are activated. At the same time, the antigen is taken up and processed into peptides by B cells that express antigen-specific B-cell receptors (BCRs, IgM and IgD), resulting in B-cell activation (signal 1) and presentation of the antigen by HLA class II molecules expressed on the B-cell surface. The B cell receives additional signals by interaction with activated antigen-specific T cells which release cytokines (signal 2), as well as through Toll-like receptors (TLRs) that bind to structures on the protein (signal 3). These signals complete the maturation process of the B cell, which undergoes antibody class-switching and somatic hypermutation with the subsequent production of high-affinity antibodies that can neutralize the antigen (e.g. BP). The T-cell dependent activation also promotes B cells to differentiate into memory cells and long-lived plasma cells (reviewed in [95,96]).

Even though recombinant human proteins should not be regarded as foreign by the immune system, factors like protein modifications (e.g. sequence modification, presence/absence of post-translational modifications) and repetitive parenteral route of administration may render them immunogenic. ADA responses to BPs could also be induced without the help from T cells, i.e. T-cell independent responses. In this scenario, activation of the B cell occurs via TLR stimulation by so called “danger signals” that can override the additional need of T-cell help (reviewed in [96,97]). A potential trigger of danger signals is the tissue damage caused by repetitive injections of BPs. In addition, protein aggregates present in the drug formulation can cross-link
BCRs and activate B-cell proliferation independent of T-cell help. Moreover, aggregates are efficiently taken up by APCs which can enhance T-cell dependent immune responses (reviewed in [97]).

In addition to these extrinsic factors, immunogenicity of BPs is also influenced by patient-related factors such as genetic variations or deficiencies, and immunological background [97]. Together, product- and patient-related factors influence an individual’s susceptibility to develop a neutralizing ADA response to BPs.

1.3.2 Antibodies against recombinant interferon beta

1.3.2.1 Immuneogenicity among interferon beta products

The immunogenicity of recombinant IFNβ was already detected during the first clinical trial with IFNβ-1b, in which 42% of the treated patients became NAb positive [98]. Development of NAbs against IFNβ was further supported by the results from trials with the different IFNβ-1a preparations, although the frequency of NAb positive patients was found to be lower for IFNβ-1a compared to IFNβ-1b [99,100]. In patients treated with intramuscular (i.m.) IFNβ-1a, NAbs were detected in 22% [100], whereas treatment with s.c. IFNβ-1a induced NAbs in 13% and 24% of the patients receiving the high dose (44µg) and the low dose (22µg) respectively [99]. This difference in immunogenicity between products has been confirmed in later studies with the NAb frequency ranging between 21-61% for IFNβ-1b [101,102], between 5-39% for s.c. IFNβ-1a at 22µg [103,104], between 13-35% for IFNβ-1a at 44µg [99,105] and between 1-13% for i.m. IFNβ-1a [104,106].

The variation in NAb frequency among products could be influenced by several factors such as route of administration (i.m. or s.c.), dosage, and injection frequency. However, results from different trials comparing the dose and administration route for a specific IFNβ product have been inconclusive (reviewed in [107,108]). Overall, it seems like the difference in immunogenicity between the IFNβ products mostly depend on the type of IFNβ molecule injected. The reason for the higher immunogenicity of IFNβ-1b is thought to be a result of its structural differences compared to endogenous IFNβ and its tendency to form aggregates, since these factors would be likely to trigger B-cell activation. For the two IFNβ-1a preparations, the higher NAb frequency for s.c. IFNβ-1a may be a result of its higher dosage frequency, which may increase the risk for B-cell activation as a result of more tissue damage. In addition, the s.c. IFNβ-1a preparation, but not the i.m. IFNβ-1a, have been reported to contain aggregates of human serum albumin (HSA) [109], which may influence its immunogenicity. In this study it was also shown that IFNβ-1b preparations have large fractions of aggregated protein that is comprised of IFNβ protein but also of HSA [109]. These findings support the hypothesis that aggregate content is a major trigger of humoral immunity to BPs. However, the likelihood for a NAb-positive patient to revert to NAb-negative status during continued treatment is higher in patients treated with IFNβ-1b than in patients treated with s.c. IFNβ-1a [110,111], suggesting that tolerance is induced earlier with the IFNβ-1b preparation. In addition, IFNβ-1b generally induce lower NAb titers compared to IFNβ-a, and patients with low titers are more likely to become NAb negative during continued treatment [104,111].

1.3.2.2 Correlation between NAb titers and IFNβ bioactivity

Most IFNβ-treated patients (~80%) develop binding antibodies (BAbs) against IFNβ, and these antibodies can be already be detected after three months of treatment [112].
Around 50% of BAb-positive patients develop NAbs, and these antibodies usually develops between 6 and 18 months of treatment [106,110]. Since NAbs interfere with the binding of IFNβ to its receptor, NAb-positive patients have reduced expression of IFNβ-inducible gene products following an IFNβ injection [113-115]. One of the most sensitive and specific biomarkers of IFNβ bioactivity is MxA [116]. The expression of MxA mRNA or protein can be used to monitor development of NAbs against IFNβ [117-119]. In patients with high NAbs, the in vivo expression of MxA in blood is significantly reduced, indicating that these patients have no or very little biological response to IFNβ. In comparison, lower NAb titers seem to have little or no effect on IFNβ bioactivity since the MxA expression is comparable to that in NAb-negative patients [113-115].

Development of NAbs is associated with reduced therapeutic efficacy, resulting in significantly increased relapse rates and MRI activity, as well as faster disease progression in NAb-positive compared to NAb-negative patients [120-123]; however, the clinical effects of NAbs are often not detectable until more than two years of treatment [120,122,124]. Instead, NAb titer levels in individual patients can be measured using different immunoassays [125] and cell-based assays [118,126,127]. This makes it possible to identify patients with NAb titers high enough to interfere with the bioactivity of IFNβ and who therefore are unlikely to respond to IFNβ therapy. Patients who lack IFNβ bioactivity because of NAb development, or who do not respond to treatment for some other unknown reason, should be switched to alternative disease-modifying drugs such as natalizumab.

However, the NAb titer level at which the IFNβ bioactivity is impaired in an individual patient is not completely established, and furthermore this is dependent on the sensitivity of the assay that is being used for NAb quantification. Study II in this thesis was conducted with the aim to identify a biologically relevant NAb-titer cut point at which the in vivo bioactivity of IFNβ is blocked and if this cut point is comparable between two different cell-based assays used for NAb titer quantification.

1.3.2.3 Patient-related factors influencing immunogenicity of IFNβ

Although product-related factors contribute to the variation in NAb frequency between IFNβ preparations, these factors cannot explain why breakage of tolerance only occurs in a subgroup of the treated patients. BAbs against IFNβ seems to develop in the majority of IFNβ-treated MS patients, but only a proportion of these patients become NAb positive later on [112]. Thus, other factors such as the patient’s genetic and immunological background are likely to influence the development of a sustained immune response. Overall, it has been shown that NAb positive patients have higher IgG antibody titers than those with BAb [128]. However, there also seems to be qualitative differences in the antibody response between these groups, as a larger fraction of the anti-IFNβ IgG antibodies are of the IgG4 subclass [129]. For B cells to produce high-affinity antibodies of the IgG class they generally require help from antigen-specific CD4+ T cells [130], and this activation is dependent on HLA class II molecules.

There is increasing evidence that immunogenicity of IFNβ is influenced by the patient’s HLA genotype. An association was shown between the DRB1*15:01-DQB1*06:02 haplotype and increased proliferative response of CD4+ T-cells to specific IFNβ epitopes in vitro [131]. Furthermore, a study on peripheral blood mononuclear cells from IFNβ-treated MS patients found an association between the
Recently, studies based on larger patient materials have associated an increased risk of developing anti-drug antibodies against IFNβ with the HLA-DRB1*04:01 and HLA-DRB1*04:08 alleles [133,134]. Also, genetic associations of an intergenic single nucleotide polymorphism (SNP) on chromosome 8q24.3 and a SNP within the HLA region, independent of DRB1*04:01 and DRB1*04:08, with the development of higher anti-drug antibody titers against IFNβ have been reported [135].

In addition to genes, life style factors might influence the risk of developing NAbs against IFNβ. Findings suggest that cigarette smoking increases the risk of developing NAbs against IFNβ (IFNβ-1a), indicating that environmental and/or life-style factors also seem to predispose to the immunogenicity of BPs [136,137]. By identifying more patient- and environmental-related factors that can influence the immunogenicity of IFNβ, it is possible that the frequency of NAbs can be reduced in the future. In study III and study IV, the patients’ OCB-status and HLA genotypes, respectively, were investigated as possible factors influencing development of NAbs against recombinant IFNβ.

1.3.3 Antibodies against natalizumab

Despite its resemblance to a human IgG4 antibody, natalizumab has been shown to be immunogenic and capable of inducing humoral immunity in treated patients. Development of antibodies against natalizumab was detected in 9% and 12% of the patients included in the first clinical trials of natalizumab [85,86]. In both studies, the majority of the positive patients (88% and 96%, respectively) already had detectable antibodies after three months of treatment. When these positive patients were followed up after at least six weeks from the first positive sample, persistent antibodies were detected in 6% of the patients, whereas antibodies had disappeared in over one third of the patients (3% and 5% respectively) [138]. When the concentration of free natalizumab in serum was measured at different time points during treatment in these patients, it was observed that antibody-positive patients had a significantly lower natalizumab concentration than antibody negative patients, indicating that these antibodies increased the drug clearance from the system. By the third month, no difference in natalizumab concentrations was found between transiently and persistently positive patients. However, after seroconversion to antibody negative status, the serum natalizumab concentration in transiently positive patients began to increase, almost reaching the levels of antibody negative patients by the end of the first year of treatment [138]. This was further supported by the finding that persistently positive patients, but not transiently positive patients, had reduced therapeutic efficacy including higher relapse rate, MRI activity and disease progression compared to antibody negative patients. In addition, persistent antibody positive patients had an increased risk of developing infusion-related adverse events [85,86,138].

Using the same enzyme-linked immunosorbent assay (ELISA) procedure as was used in the first clinical studies, later observational studies have found that anti-natalizumab antibodies develop in 4.5-14% of treated patients, of whom 1.6-9% have been persistently positive and 1-5% have been transiently positive [87,139,140]. Other studies have also found that anti-natalizumab antibodies develop early, with most patients becoming positive during the first six months of treatment [87,139].

The transient antibody response to natalizumab observed in a subgroup of patients suggests that some patients become tolerant to natalizumab during continued treatment.
Since antibody development against natalizumab is an efficacy and safety issue in treated patients, it is of importance to find biomarkers to enable the identification of individual patients at risk of becoming persistently antibody positive. In study V of this thesis, the aim was to search for an antibody profile that would make it possible to distinguish transiently and persistently positive patients, in order to predict at an early stage whether a patient should discontinue treatment or not.
2 AIMS OF THE THESIS

The overall aims of this thesis were to determine the prevalence and biological effects of ADA against IFNβ and natalizumab, and to identify factors that influence the immunogenicity of IFNβ in patients with MS.

The specific aims of each study were the following:

Study I:
To assess how the prevalence of NAbs, the levels of NAb titers and the IFNβ preparations used for treatment of MS patients changed in 2009-2010 compared to 2003-2004 when monitoring NAbs against IFNβ became clinical practice in Sweden.

Study II:
To evaluate how well NAb titers against IFNβ correlate between the two bioassays MxA induction assay and iLite, and to determine at which titer level the in vivo biological activity of IFNβ is impaired in treated MS patients.

Study III:
To compare the development of NAbs against IFNβ between MS patients with and without OCB in the CSF in order to determine if OCB status influences the risk of developing NAbs against IFNβ.

Study IV:
To investigate whether HLA genes influence the risk of developing NAbs to IFNβ, and if so, whether the genetically determined risk of developing NAbs varies depending on type of IFNβ preparation.

Study V:
To characterize the ADA response against natalizumab and to investigate differences in the antibody response between patients who develop persistent and transient antibodies against the drug.
3 MATERIALS AND METHODS

More detailed information is found in the “Material and Methods” sections in the original articles.

3.1 PATIENTS AND STUDY DESIGN

Informed consent, oral or written, was obtained from patients participating in research and all studies were approved by the regional ethical committee in Stockholm. Patients with a diagnosis of MS, according to McDonald criteria [8], analyzed for NABs against IFNβ between 2003 and 2013, or screened for anti-natalizumab antibodies between 2006 and 2011 in the routine NAb laboratory at Karolinska Institutet in Stockholm, were included in the works of this thesis. Laboratory test results, clinical data and information on treatment of relevance for the respective studies were obtained from the NAb registry and Swedish Multiple Sclerosis (SMS) registry (http://www.msreg.net).

HLA genotype data were obtained from a genetic database containing genotype data from different genetic projects on MS patients in Sweden: EIMS (Epidemiological Investigation of Multiple Sclerosis) or GEMS (Genes and Environment in Multiple Sclerosis), STOP MS (Stockholm Prospective Assessment of Multiple Sclerosis), and IMSE I (Immunomodulatory Multiple Sclerosis study I).

Study I included all (n=1296) IFNβ-treated Swedish and Icelandic MS patients who had at least one serum sample analyzed for NABs during 2009 and 2010. The frequency of NAb-positivity and distribution of titer levels, usage of different IFNβ preparations and treatment duration in this cohort were compared with previously published data on 1115 MS patients analyzed for NABs during 2003 and 2004 [104]. In both study cohorts, only the result from the first analyzed sample was included for each patient.

In study II, 44 IFNβ-treated MS patients, who had been previously analyzed for NABs, were recruited from four neurological hospital centers in Sweden, including Karolinska University Hospital in Huddinge and Solna, Danderyd Hospital, and Sahlgrenska University Hospital, between 2010 and 2013. Patients were asked to participate on the basis of their previous NAb status in order to include patients with NAb titers ranging from negative to very high positive. After written informed consent was signed by all participants, peripheral blood samples were collected for isolation of RNA and serum to be used for mRNA and protein expression studies on IFNβ-induced biomarkers, and for NAB analyses.

In study III, the included patients were assayed for NABs between 2003 and 2009. Information regarding NAb status and IFNβ preparation use was available for 2070 OCB-positive and 149 OCB-negative MS patients. Of these patients, date of treatment onset was recorded for 1886 OCB negative and 137 OCB positive, and HLA-DRB1 genotype data was accessible for 506 OCB positive and 26 OCB negative.

In study IV, data on all HLA-genotyped MS patients who had been assayed for NABs against IFNβ between 2003 and 2012 were obtained. After excluding patients of non-Scandinavian origin (n=403) to reduce population heterogeneity and patients who did not fulfill the criteria for NAB positivity and negativity (n=420), the study comprised 364 NAb positive and 539 NAb negative patients. If several sources of a HLA-
genotype were found for one patient, the data obtained from classical genotyping was used. Carriage of HLA-DRB1*04:01 and *04:04 was identified from imputed genotypes. Binding of specific HLA alleles to the two types of recombinant IFNβ molecules, IFNβ-1 (166 amino acids) and IFNβ-1b (165 amino acids) was predicted using the NetMHCII and NetMHCpan servers from the free on-line prediction services at CBS (http://www.cbs.dtu.dk/services/).

In Study V, we had access to all Swedish natalizumab-treated MS patients who had been screened for anti-natalizumab antibodies between August 2006 and September 2011. Patients who had not pre-treatment sample were excluded, resulting in a study cohort comprising 1391 patients and a total of 5425 samples. Information on demographic and clinical characteristics of the patients was available for 888 patients, who were found to be representative for the total study cohort with regard to age, gender, treatment duration and total anti-natalizumab antibody responses.

3.2 NEUTRALIZING ANTIBODY ASSAYS

Detection of IFNβ-specific antibodies with capacity to neutralize the biological responses to IFNβ can be performed using different in vitro bioassay methods. In this thesis, both screening and quantitation of IFNβ therapy-induced NAbs has been performed using two different cell-based assays, the MxA induction assay and the luciferase reporter gene assay iLite. Both assays are semi-quantitative, which means that the neutralizing capacity of a serum is not given in absolute amount but relative to measurements from IFNβ standard curves.

In both assays, serum samples isolated from peripheral venous blood, preferably collected at least 36 hours after IFNβ injection, were used. For the iLite assay, patient’s sera were heated for 30 minutes at 56°C to inactivate complement, whereas no pretreatment was required for the MxA assay [141]. Samples were stored at -20°C and/or -80°C before analysis.

3.2.1 MxA induction assay

The MxA induction assay for measurement of NAbs against IFNβ is a modification of the MxA induction assay developed for measuring bioactivity of type I IFNs in vitro [118,141]. In this NAb assay, neutralization of IFNβ-induced expression of MxA can be measured both at the protein level by ELISA [118,141] and at the mRNA level by quantitative real-time polymerase chain reaction (RT-PCR) [117]. For the work of this thesis, the MxA protein induction assay was employed for samples analyzed from 2003 to 2006, as described by Sominanda et al. [104], while samples from 2007 and onwards were analyzed with the MxA gene expression assay (MGA) [117].

In both assays, patient serum diluted in a fixed concentration of IFNβ (10 international units [IU]/ml of i.m. IFNβ-1a) is added to IFNAR expressing cells (lung carcinoma cell line A549 from ATTC). In the presence of IFNβ-specific NAbs, the serum will inhibit the interaction between IFNβ and its receptor, which prevents induction of type I IFN-specific gene expression, e.g. MxA. The serum samples’ neutralizing activity, i.e. NAb titer, is defined as the serum dilution at which 10% (1 IU/ml) of the IFNβ bioactivity remains. To determine at which serum dilution this ten-fold reduction occurs, a standard curve of recombinant IFNβ-1a, serially diluted from 10 to 0 IU/ml, is added to A549. The dose-dependent induction of MxA is then used to calculate the quantity of MxA expressed when cells are stimulated with 1 IU/ml
of IFNβ, also known as EC₅₀, which is the linear region of the sigmoidal dose-response curve. The EC₅₀ value generated from the standard curve is then used to assess at which dilution a patient serum neutralizes the IFNβ activity from 10 to 1 IU/ml. The reciprocal of this dilution is used to define the NAb titer, as described in section 3.2.3.

3.2.2 Luciferase reporter gene assay

In study II, NAb analyses were performed using both the MxA gene expression assay described above, and the iLite™ anti-human IFNβ bioassay from Biomonitor Limited. In the iLite assay, IFNβ bioactivity is measured using the cell-cycle arrested human cell line PIL5, which derives from the work done by Lallemand and colleagues [126,142]. These type I IFN-sensitive cells carry the firefly luciferase gene under the control of an IFN-responsive promoter element. In the absence of NAbs, IFNβ binds to its receptor and stimulates the expression of luciferase. The level of IFNβ-induced luciferase expression is determined through the protein’s enzymatic activity, which is measured as strength of the bioluminescence signal [126].

As in the MxA induction assay, the neutralizing activity of patient’s serum is measured as its ability to reduce the activity of the added IFNβ from 10 to 1 IU/ml. To determine the luciferase activity that is induced by 1 IU/ml of IFNβ, the relative luminescence unit (RLU) is plotted against an IFNβ standard curve diluted from 80 to 0 IU/ml. The level of luminescence measured at the midpoint of the linear part of the dose-response curve corresponds to 1 IU/ml of IFNβ activity, and this RLU value is then used to determine the dilution of patient’s serum that reduces the IFNβ activity from 10 to 1 IU/ml. The reciprocal of this dilution is used to define the NAb titer, as described in section 3.2.3.

3.2.3 Calculation of neutralizing antibody titers

After titration of samples, NAb titers (t) are calculated using the Kawade-Grossberg formula [143-145]:

\[ t = \frac{f}{n-1} / (10-1) \]

Where \( f \) = reciprocal of serum dilution at 1 IU/ml, and \( n \) = the ratio of added IFNβ to remaining IFNβ in the sample. Using this formula, NAb titers are expressed as ten-fold reduction units per milliliter (TRU/ml), which is recommended by the World Health Organization [144] and the European Federation of Neurological Societies task force [107]. It has been shown that NAb titers adjusted according to the Kawade formula and expressed as TRU/ml are comparable, although different cell-based assays were used to detect the neutralizing antibody titer [126,146].

3.2.4 Classification of neutralizing antibody status

In all studies (study I, II, III and IV), patient serum samples were considered negative if NAbs were undetectable in the screening assay, and if NAb titers were <10 TRU/ml in the titration assay. Patients with a NAb titer ≥10 TRU/ml were defined as positive. In study I and II, NAb positive samples were categorized as shown in Table 1.

**Table 1. NAb titer classification.**

<table>
<thead>
<tr>
<th>Titer category</th>
<th>Study I</th>
<th>Study II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low positive</td>
<td>10-49 TRU/ml</td>
<td>10-50 TRU/ml</td>
</tr>
<tr>
<td>Medium positive</td>
<td>50-199 TRU/ml</td>
<td>51-200 TRU/ml</td>
</tr>
<tr>
<td>High positive</td>
<td>&gt;200 TRU/ml</td>
<td>&gt;200 TRU/ml</td>
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</table>
In study II and IV, a titer of 150 TRU/ml was used as a cut-point for biologically relevant titers, as in vivo MxA expression following IFNβ administration has shown to be markedly reduced at this titer level [113].

### 3.3 MEASUREMENTS OF INTERFERON-BETA BIOMARKERS

The biological effect of NAbs can be determined by measuring the in vivo bioactivity of IFNβ following an injection of the drug. In the presence of NAbs there will be a titer-dependent reduction in the expression of IFNβ-induced genes such as MxA [119,128]. In study II, the in vivo IFNβ-induced expression of MxA and CXCL (C-X-C motif chemokine)-10 mRNA in whole blood, and CXCL-10 protein in serum, was measured to assess the biological response to IFNβ therapy at different NAb titers.

Total RNA was isolated from 3 ml peripheral venous blood collected 8 to 18 hours after an IFNβ injection. RNA quality and concentration was determined before total RNA was reversely transcribed into cDNA. Taqman RT-PCR was performed to determine MxA and CXCL-10 mRNA expression. Relative gene expression of MxA and CXCL-10 was calculated using the $2^{\Delta\Delta C_{T}}$ method [147], where expression levels were normalized to the reference gene HPRT1 (hypoxanthine-guanine phosphoribosyltransferase) and further calibrated against the expression of healthy controls.

Serum was isolated from 5 ml peripheral venous blood collected both 8-18 hours and at least 36 hours after IFNβ injection. Concentration of CXCL-10 was measured using the human CXCL10/IP-10 Quantikine ELISA kit from R&D Systems, according to manufacturer’s protocol.

### 3.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

#### 3.4.1 Quantification of total IgG

The concentration of IgG antibodies was measured in serum samples collected before first natalizumab infusion from 64 MS patients. Total IgG was quantified using the human IgG ELISA kit (ALP) from Mabtech.

#### 3.4.2 Natalizumab-specific antibody detection

The total antibody response to natalizumab was determined by a bridging ELISA method and performed according to the standardized protocol developed by BiogenIdec. In brief, patient serum and control samples were added to natalizumab-coated microtiter plates in the presence and absence of competing soluble natalizumab. Captured natalizumab-specific antibodies were detected using biotinylated natalizumab and streptavidin-horseradish peroxidase, with subsequent addition of tetramethylbenzidine substrate and sulfuric acid ($H_2SO_4$), before optical density (OD) was measured at 450 nm.

Wells containing serum samples with soluble natalizumab (competition well) were used to confirm the binding specificity of captured antibodies detected in wells containing serum samples without soluble natalizumab (detection well). Samples with at least twice as high reactivity against natalizumab in the detection well compared to the competition well were considered positive, meaning that an OD ratio $\geq 2.0$ was used as cut-point for antibody positivity.
3.4.2.1 Detection of natalizumab-specific IgM and IgG1-3 antibodies

For detection of natalizumab-specific antibodies of the IgM class and the subclasses IgG1, IgG2 and IgG3, slightly modified versions of the screening ELISA described above were used. Natalizumab-specific antibodies of the IgG1-3 subclasses were detected using biotinylated monoclonal mouse anti-human IgG1-3 antibodies respectively. Natalizumab-specific antibodies of the IgM class were captured to microtiter plates coated with an anti-human IgM antibody, followed by detection according to the standardized protocol described above. Patient sera positive or negative for anti-natalizumab IgM or IgG1–3 antibodies were included as controls on all plates, and results were reported as OD ratios as described above.

3.4.2.2 Detection of natalizumab-specific IgG4 antibodies

Detection of natalizumab-specific antibodies of the IgG4 subclass was performed using an ImmunoCAP assay developed by Phadia. In this immunoassay, patient serum was added to ImmunoCAP tests coupled with F(ab’)2 fragments of natalizumab. Captured natalizumab-specific IgG4 antibodies were detected using the instrument Phadia 100. A concentration of 0.125 mg Antigen/liter was used as cut-point for positivity, which was based on the mean value + 2 standard deviations of 18 healthy control donors.

3.5 GENOTYPING

Classical genotyping for the HLA-A, HLA-C and HLA-DRB1 genes was performed using kits from Olerup SSP AB [148] and for the HLA-B gene, a Luminex-based reverse SSO method was used. HLA-genotypes were also obtained through imputation, either with genotypes from the IMSGC WTCCC2 MS genome wide association study [33] or from the Immunochip [149], using HLA*IMP:01 [150] and HLA*IMP:02 [151] respectively.

3.6 CEREBROSPINAL FLUID (CSF) ANALYSIS

CSF analyses were performed during the diagnostic workup for each patient. Oligoclonal IgG bands (OCB) were detected in paired CSF and plasma samples using isoelectric focusing followed by IgG-specific immunolabelling [152]. Increased intrathecal IgG levels were measured using the IgG index (IgG_{CSF}/IgG_{plasma})/(albumin_{CSF}/albumin_{plasma}), which correct for possible dysfunction of the blood-CSF barrier [153]. In study III, OCB positivity was defined as having two or more OCB in CSF that differ from plasma and/or increased intrathecal synthesis of IgG, according to the McDonald criteria [8].

3.7 STATISTICS

All reported probability, p, values were based on two-sided statistical tests and considered significant if below an alpha-level of 0.05. Analyses were performed using GraphPad Prism from GraphPad Software Inc., USA, or the free software package R [154].

Patient characteristics were presented as mean or median, with ± SEM (standard error of mean), IQR (interquartile range) or min-max range, for continuous variables and as absolute numbers and frequencies for categorical variables.

The Shapiro-Wilk test was used to verify normal distribution of the data. The two-sample t-test was used when analyzing unpaired data with normal distribution. When
normality was not assumed, the non-parametric Mann-Whitney test was used for unpaired data, whereas the Wilcoxon-matched-pairs signed rank test was used for comparisons between paired data. Furthermore, the non-parametric Spearman correlation test was used to determine relationships between two variables.

For analyses of categorical data, the Fisher's exact test or chi-square test, with or without Yates’ correction, were used for comparisons between two groups, while the Kruskal-Wallis test with Dunn’s multiple comparison was used for comparisons between three groups. In study I, a Cochran-Mantel-Haenszel test was used to determine the overall significance of NAb development between the study cohorts when controlling for the covariates, i.e. the different IFNβ preparations.

Correction for multiple testing was done with the Bonferroni method to compensate for the number of comparisons made. This was done in studies I and IV to control the probability of false positive findings.

Logistic regression analyses were performed to control for confounding effects and interaction between the variables OCB status and IFNβ preparation on NAb outcome in study III.

Odds ratio (OR) calculations were performed to measure the strength of association between OCB status and NAb positivity (study III), and between HLA allele group carriage and NAb positivity and development of biologically relevant titers (study IV).

Absolute risk (AR) was calculated in study IV for development of NAbs and biologically relevant titers to measure the influence of HLA allele group carriage within each of the three treatment groups. The AR was estimated using Bayes’ theorem [155]. In this formula, the AR of reaching the outcome (NAb positivity/high titers) is based on the proportion of patients in the Swedish NAb registry that achieved these outcomes within each treatment group (i.e. the proportion of patients that developed NAbs and high NAb titers for each preparation).
4 RESULTS AND DISCUSSION

4.1 STUDY I

Routine analysis for NAb against IFNβ became available in Sweden in 2003 and soon after that the monitoring of NAb became clinical practice. In a previous study that included 1115 Swedish and Icelandic MS patients treated with IFNβ, who were routinely analyzed in 2003-2004 for the presence of NAb, 32% of the patients were identified as NAb positive and high antibody titers above 200 TRU/ml were present in as many as 16% of the whole study group [104].

In this study we investigated whether the prevalence and titer levels of NAb as well as the distribution of IFNβ preparations used had changed from when the routine monitoring started in 2003-2004 compared to 2009-2010.

4.1.1 Decreased seroprevalence over time

When comparing the data from the 1296 IFNβ-treated MS patients from Sweden and Iceland that were routinely analyzed for NAb between 2009 and 2010 with the previously published data that included 1115 patients, the overall seroprevalence of NAb had decreased markedly from 32% to 19% between 2003-2004 and 2009-2010 (Table 2). Interestingly, a decrease in NAb positivity was only seen in patients treated with the IFNβ-1a preparations whereas the percentage of NAb positive patients among those receiving IFNβ-1b remained constant (Table 2).

Table 2. Seroprevalence of NAb in 2003-2004 compared to 2009-2010.

<table>
<thead>
<tr>
<th></th>
<th>2003-2004</th>
<th>2009-2010</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Distrib. (%)</td>
</tr>
<tr>
<td>All patients</td>
<td>1115</td>
<td>n/a</td>
</tr>
<tr>
<td>i.m. IFNβ-1a</td>
<td>257</td>
<td>23</td>
</tr>
<tr>
<td>s.c.IFNβ-1a (22)</td>
<td>276</td>
<td>25</td>
</tr>
<tr>
<td>s.c.IFNβ-1a (44)</td>
<td>294</td>
<td>26</td>
</tr>
<tr>
<td>IFNβ-1b</td>
<td>288</td>
<td>26</td>
</tr>
</tbody>
</table>

* Corrected P-values are reported for NAb frequency comparisons of drug preparations between 2003-2004 and 2009-2010.
† A Cochran-Mantel-Haenszel chi-squared test yielded a p-value of 1.5x10^-5 across the treatments.
n/a=not applicable, NS=not significant

4.1.2 Changes in NAb titer levels over time

Moreover, there was a change in NAb titer levels between 2003-2004 and 2009-2010 and the greatest, and most important, change occurred in the group of patients with high NAb titers, where a decrease from 16% to 7% was seen. After stratification based on titer levels and IFNβ preparations, a significant decrease was only noted in the high titer group of patients receiving IFNβ-1a (Avonex and Rebif 44).

When comparing the proportion of seropositive patients over time the overall seroprevalence had decreased for all time points investigated (Figure 1). A difference in seropositivity could mainly be seen for patients that had been on treatment for 4-5 years, as the seroprevalence decreased from 41% to 13% between 2003-2004 and 2009-2010 (Figure 1). Interestingly, when looking at the different NAb titer categories, the proportion of patients with low positive titers was higher for all time points in 2009-2010 while the proportion with high titers was lower compared to 2003-2004. The
higher proportion of low titer positive patients might indicate that patients with low positive titers are recommended to stay on IFNβ treatment since these NAb levels are unlikely to suppress the therapeutic efficacy.

**Figure 1.** Treatment duration and NAb seroprevalence in 2003-2004 and 2009-2010.

4.1.3 Discussion

Together these results suggest that the incorporation of routine NAb testing into clinical practice, and the fact that clinicians seem to take these results into consideration when making treatment decisions, has had an effect on NAb seroprevalence and titer levels in Sweden and Iceland during the five years between the studies. In addition, the reason(s) for the lower proportion of NAb positive patients, especially those with high antibody levels, may also be the preferential use of less immunogenic products or improvements of drug formulations that result in lower immunogenicity against IFNβ. There is general agreement that i.m. IFNβ-1a is the least immunogenic preparation, and in this study we observed that the proportion of patients treated with this product had increased significantly between the periods, from 23% in 2003-2004 to 45% in 2009-2010. We could also see that the use of the lower dose (22µg) of s.c. IFNβ-1a, which has been suggested to be more immunogenic than the high dose preparation, had decreased substantially and this might be reflected in the overall reduction in NAb frequency.

Furthermore, the decreased frequency of NAb positive patients might be a result of the efforts by the pharmaceutical companies to produce IFNβ preparations with lower immunogenicity. The lower proportion of NAb-positive patients in the group of patients treated with s.c. IFNβ-1a might be explained by the introduction of a less immunogenic formula, the Rebif New Formulation (RNF), in Sweden in October 2007 [156]. It is also possible that patients treated with i.m. IFNβ-1a in 2003-2004 had received prior treatment with the earlier and more immunogenic formulation [157], which might account for the observed reduction of NAbs in patients receiving i.m. IFNβ-1a.

Finally, the use of different methods to detect and quantify NAb had changed between the periods. It is possible that the MxA gene expression assay used in the later study is less sensitive than the MxA protein assay. However, the constant proportion of NAb-positive patients in the IFNβ-1b treated group speaks against this explanation.
4.2 STUDY II

The objective of this study was to quantify and compare IFNβ NAb titers analyzed with the MxA induction assay, MGA, and the luciferase induction assay, iLite, and to correlate respective NAb titer readouts with expression levels of the IFNβ-induced genes MxA and CXCL-10.

Of the 44 IFNβ-treated MS patients analyzed in this study, 8 received i.m. IFNβ-1a, 11 received s.c. IFNβ-a and 25 received s.c. IFNβ-1b. Serum samples for NAb analysis were available for all 44 patients, and these samples were collected 11-162 hours (mean 70 hours) after the latest IFNβ injection. NAb positivity was defined as a titer ≥10 TRU/ml in both assays. In MGA, 28 patients (64%) were classified as positive with titers between 10 and 6467 TRU/ml, compared to 21 patients (48%) in iLite with titers between 11 and 11584 TRU/ml. Although 7 patients with low titers in MGA were negative in iLite, and 8 patients with medium titers in MGA were either low positive (n=2) or high positive (n=1) in iLite (Table 3), we found that NAb titers strongly correlated between assays (Spearman r=0.94, 95% CI 0.88-0.97).

Table 3. Comparison of NAb titers against IFNβ measured by MGA and iLite.

<table>
<thead>
<tr>
<th>MGA, TRU/ml</th>
<th>iLite, TRU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10 (n=23)</td>
</tr>
<tr>
<td>&lt;10 (n=16)</td>
<td>16</td>
</tr>
<tr>
<td>10-50 (n=10)</td>
<td>7</td>
</tr>
<tr>
<td>51-200 (n=8)</td>
<td>0</td>
</tr>
<tr>
<td>&gt;200 (n=10)</td>
<td>0</td>
</tr>
</tbody>
</table>

MGA = myxovirus resistance protein A gene expression assay; TRU/ml = ten-fold reduction units per milliliter

4.2.1 Biologically functional NAb titer cut-point

Bioactivity of IFNβ at different NAb titers was assessed by measuring the *in vivo* IFNβ-induced MxA and CXCL-10 gene expression 8-18 hours (mean 11.6 hours) post-IFNβ injection in 42 patients. There was significant negative correlations between MxA and CXCL-10 mRNA expression and patients’ NAb titers measured with both MGA (MxA: Spearman r= -0.88, 95% CI -0.94 to -0.79; CXCL-10: Spearman r= -0.91, 95% CI -0.95 to -0.83) and iLite (MxA: Spearman r= -0.89, 95% CI -0.94 to -0.81; CXCL-10: Spearman r= -0.90, 95% CI -0.94 to -0.81).

To evaluate whether the serum level of CXCL-10 also is a sensitive biomarker for IFNβ response, CXCL-10 concentrations were measured in 32 serum samples collected 8-14 hours (T1) and ≥ 36 hours (T2) post-IFNβ injection. At both time points, there was a significant negative correlation between CXCL-10 levels and NAb titers measured with MGA (T1: Spearman r= -0.83, 95% CI -0.92 to -0.66; T2: -0.57, 95% CI -0.78 to -0.25) and iLite (T1: Spearman r= -0.88, 95% CI -0.94 to -0.77; T2: -0.74, 95% CI -0.87 to -0.52). However, CXCL-10 levels were significantly reduced between T1 and T2 in (mean 468 pg/ml vs. 235 pg/ml, p < 0.0001), indicating that serum CXCL-10 is a sensitive biomarker for responsiveness to IFNβ if measured around 8-14 hours after IFNβ injection.

Our group has previously shown that the *in vivo* bioactivity of IFNβ is reduced by 80% in patients with NAb titers >150 TRU/ml compared to NAb negative patients, when
NAb titers were measured with the MxA protein induction assay [113]. When using the same biological cut-point for NAb titers determined by the MGA and iLite assay, we found that both MxA and CXCL-10 mRNA expression was reduced by 97% in NAb positive compared to negative patients (Figure 2). Thus, a medium NAb titer of 150/TRU/ml indicates complete neutralization of the in vivo response to IFNβ therapy, and can be used as a biologically functional cut-point for both assays.

**Figure 2.** Relative mRNA expression (RQ) of MxA and CXCL-10 in patients with different NAb titers measured by the MGA and iLite assay.

### 4.2.2 Discussion

The MxA gene expression induction assay (MGA) has been used for screening and quantification of NAbs against IFNβ in the NAb laboratory at Karolinska Institutet since 2006-2007. This bioassay requires high maintenance and is time consuming to perform; therefore we were looking for a more easy-to-perform bioassay, capable of both detecting and quantifying NAbs with similar sensitivity and accuracy to MGA. From these results, we conclude that the iLite bioassay can replace our in-house MxA induction assay for routine monitoring of NAbs against IFNβ. Furthermore, a medium NAb titer of 150 TRU/ml seems to be a good cut-point to define a significant reduction or total absence of IFNβ bioactivity in treated MS patients, since the IFNβ-induced expression of both MxA and CXCL-10 was completely neutralized over this cut-point.

We observed that the MGA assay has somewhat higher sensitivity to detect low NAb titers compared to iLite, as shown by the difference in classification of seven serum samples. However, this is not considered to be a significant problem, since NAb titers in the lower ranges seem to have minor effect on the in vivo biological response to IFNβ.

Although there have been some controversies regarding the clinical relevance of NAbs, it seems unlikely that therapeutic efficacy would be maintained even though the in vivo expression of type I IFN biomarkers, e.g. MxA and CXCL-10, is almost completely inhibited in patients with high NAb titers. Interestingly, the CXCL-10 expression was unexpectedly low in a subgroup of the NAb-negative patients, regardless of which assay that was used to establish the NAb status (Figure 2). Non-antibody mediated neutralization of IFNβ bioactivity has been observed in a small percentage (~4%) of
IFNβ-treated MS patients [158]. These patients may represent a subgroup of patients who are non-responders to IFNβ even in the absence of NAbs. Thus, these patients possibly need to shift to non-IFNβ therapy.

It is important to remember that differences in the detection and quantification of NAbs against IFNβ depend not only on type of assay used in clinical monitoring, but also on variations in assay performance between different laboratories [159]. Thus, a cut-point of 150 TRU/ml may not be the correct cut-point for other types of NAb assay methods, and not even for the iLite assay if used in other laboratories where NAb monitoring is performed. For results to be comparable between different laboratories and clinical studies, it would be necessary to agree upon an assay to be used for NAb monitoring and to validate this method according to standard protocols.
4.3 STUDY III

OCB-positive and OCB-negative MS patients have been shown to constitute two immunogenetically distinct subgroups of MS since they are associated with different HLA-DRB1 alleles, HLA-DRB1*15 and HLA-DRB1*04, respectively [23,24]. The reason for the absence of these MS characteristic IgG antibodies in a minor proportion of MS patients is unidentified. In this study we were interested in investigating the hypothesis that a potential alteration in the humoral immune response between these two patient subgroups would result in different propensities of developing NAb against IFNβ.

4.3.1 Association between NAb outcome and OCB status

Of the 2219 MS included patients for whom we had information on OCB status, NAb status and treatment use, OCB positivity was 93% (n=2070), which is within the range of what has previously been reported (reviewed in [9]). When comparing the proportion of NAb positive patients between the groups, OCB-negative patients were found to develop NAbs against IFNβ to a significantly lesser extent compared to OCB-positive patients (19% versus 28%, p=0.02). Since treatment duration is a known risk factor for NAb development the time from treatment onset to collection of NAb samples was compared between OCB-negative and OCB-positive patients as well as between NAb-negative and NAb-positive patients. However, treatment duration did not differ between the groups and was thus considered to have no impact on the observed difference in NAb seroprevalence between patients with and without OCB.

4.3.2 Differences in immunogenicity of interferon beta-1a

Another factor known to affect NAb development is the type of IFNβ preparations used, with IFNβ-1b generally being more immunogenic than IFNβ-1a (reviewed in [160]). Therefore, we also sought to determine whether the immune response to the different IFNβ preparations, IFNβ-1a and IFNβ-1b, differed between the OCB-positive and OCB-negative group. Interestingly, when stratifying the study group for both OCB status and IFNβ preparation, we found that OCB-negative patients receiving treatment with IFNβ-1a were significantly less likely to become NAb positive compared to OCB-positive patients receiving IFNβ-1a (p=0.005), whereas no difference in NAb positivity was seen for those receiving IFNβ-1b (p=0.86) (Figure 3).
4.3.3 Possible influence of HLA allele carriage

These unexpected results led us to the hypothesis that the antigen presenting ability might differ between the groups, resulting in lower immune activation and antibody production against IFNβ-1a in OCB-negative patients. This hypothesis was also supported by the fact that the HLA class II alleles HLA-DRB1*04:01 and *04:08 recently had been associated with increased risk of developing NAbs to IFNβ [134]. Unfortunately, data on HLA-DRB1 allele carriage was only accessible for 532 included patients, which was too few to investigate whether our finding could be affected by differences in DRB1 carriage. However, we could confirm the previously reported associations to distinct HLA-DRB1 alleles for the two MS subgroups, HLA-DRB1*15 for OCB-positive MS and HLA-DRB1*04 for OCB-negative MS. The accuracy of these results is strengthened by a recent publication confirming the association of OCB status to these distinct HLA alleles [22].

4.3.4 Confounding factor

A possible confounding factor in our study is that CSF analyses performed at different laboratories could differ in sensitivity to detect OCB. However, the proportion of OCB-negative patients does not differ from that seen in previous studies; thus it seems less likely to be the cause of a biased selection. Even though some samples might have been falsely detected as OCB negative, this would at worst reduce the association seen to the OCB-negative group, and should not undermine our positive findings.

4.3.5 Discussion

In conclusion, the results from this OCB phenotype-NAb status analysis show that therapeutic IFNβ, more specifically the IFNβ-1a preparations are less immunogenic to, or maybe more immunologically tolerated by OCB-negative compared to OCB-positive MS patients. Thus, we have identified an additional patient-related factor that affects the development of NABs in IFNβ-treated MS patients.

Absence of OCB and the less frequent immunological response towards therapeutic IFNβ might imply that more extensive immunological differences between OCB-negative and OCB-positive patients exist. Whether there are other alterations in the
humoral immune response between these two groups of MS patients would be very interesting to investigate. For example, this could be investigated by looking at differences between the groups in their IgG responses following vaccinations and against common herpes viruses such as cytomegalovirus (CMV), EBV, HHV-6 and varicella zoster virus (VZV). A recent finding that these two subgroups of MS differ genetically, not only in HLA genes [22], could indicate that clinical differences seen in previous studies depend on the different genetic backgrounds in these patients. Absence of OCB might reflect that these patients constitute a subpopulation of MS with a specific immunological phenotype that results in less aggressive immunological responses, both in terms of MS pathogenesis and in a broader sense.
4.4 PAPER IV

Recent investigations have connected the immunogenicity of IFNβ to specific HLA-DRBI alleles [133-135]. In this study, we investigated if HLA class I and class II genes are associated with the susceptibility to develop NAbs as well as NAb titers high enough to be biologically relevant. We used a Swedish cohort of 364 NAb positive and 539 NAb negative MS patients treated with IFNβ.

4.4.1 HLA allele groups associated with NAb development

Overall, we found that HLA-DRBI*15 carriage both increased the risk of developing NAb (OR 1.43, 95% CI 1.17-1.74) and biologically relevant titers (OR 1.58, 95% CI 1.25-2.0). For HLA-B*07 carriers the risk of developing biologically relevant titers was increased six-fold compared to non-carriers. Contrary to previous studies [133-135], the overall carriage frequency of the HLA-DRBI*04 allele group did not differ between NAb positive and NAb negative patients (NAb development: OR 1.08, 95% CI 0.83-1.39; biologically relevant titers: OR 1.21, 95% CI 0.9-1.63).

4.4.2 Interferon beta preparation-dependent HLA associations

Since the immunogenicity of IFNβ is recognized to vary among preparations, we continued to investigate how NAb development is influenced by HLA carriage in combination with the different types of IFNβ preparations. Based on the preparation used at the time of NAb analysis, patients were stratified into the treatment groups intramuscular IFNβ-1a (n=346), subcutaneous IFNβ-1a (n=355) and IFNβ-1b (n=196). Using this approach, associations to distinct HLA allele groups was observed for the different treatment groups, HLA-DRBI*04 for IFNβ-1b and HLA-DRBI*15 for both types of IFNβ-1a preparations. Interestingly, we observed a trend towards reduced risk of developing biologically relevant NAb titers in HLA-DRBI*15 carriers receiving IFNβ-1b (OR 0.33, nominal p value=0.0052, BF corrected p value=0.098).

Since an association to the previously reported risk allele HLA-DRBI*04 was only observed in patients receiving IFNβ-1b in our cohort, we performed subgroup analysis of HLA-DRBI*04 alleles in these patients. This analysis revealed that HLA-DRBI*04:01 carriers have an increased risk of developing NABs (OR 3.43, 95% CI 1.48-7.93). However, an association to the HLA-DRBI*04:04 allele, which has been nominally associated to reduced risk of developing anti-IFNβ antibodies [133,134], could not be replicated in our cohort.

The different genetic predispositions to NAb development depending on type of IFNβ preparation made us examine whether these HLA alleles were more or less likely to bind to any specific IFNβ-1a or IFNβ-1b peptides that could explain the distinct associations. The only observed difference in the binding analysis was that HLA-DRBI*04:01 was more likely to bind certain IFNβ-1b peptides compared to the non-associated alleles HLA-DRBI*04:04 and HLA-DRBI*15.

Calculation of absolute risk was used to estimate how carriage of HLA-DRBI*04 and HLA-DRBI*15 impact the risk of developing NABs and biologically relevant titers beyond the risk given by the treatment itself. This showed that carriage of HLA-DRBI*15 increases the risk substantially for both outcomes in s.c. IFNβ-1a treated patients, and to a lesser extent in those receiving i.m. IFNβ-1a, whereas carriage of HLA-DRBI*15 lower the risk in patients receiving IFNβ-1b. In HLA-DRBI*04
carriers, the reversed relationship was observed (Table 4). Furthermore, the absolute risk for NAb development and biologically relevant titers was constantly lower for i.m. IFNβ-1a compared to the s.c. IFNβ-1a and -1b preparations, irrespective of whether the risk alleles HLA-DRB1*04 and HLA-DRB1*15 were present or absent.

Although HLA-DRB1*15 predisposes to development of NAbs against the two IFNβ-1a preparations, which contain identical IFNβ molecules, our results also indicate that product-related factors greatly affect immunogenicity. This is demonstrated by the difference in NAb prevalence, which is considerably lower in patients receiving i.m. compared to s.c. IFNβ-1a. For example, more frequent injections of higher doses into the skin, which harbors high numbers of professional antigen-presenting cells that will encounter the antigen [161], are more likely to trigger an immune response compared to less frequent low-dose injections in the muscles. The combination of these factors makes i.m. IFNβ-1a the least immunogenic preparation, regardless of genotype carriage.

**Table 4.** Absolute risk analysis with IFNβ preparation and presence or absence of HLA-DRB1*04 and HLA-DRB1*15 as factors influencing the susceptibility of developing NAb and biologically relevant titers of NAb.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><strong>DRB1*04 positive</strong></th>
<th><strong>DRB1*04 negative</strong></th>
<th><strong>DRB1*15 positive</strong></th>
<th><strong>DRB1*15 negative</strong></th>
<th>Overall frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.m. IFNβ-1a</td>
<td>6.7</td>
<td>9.9</td>
<td>11.3</td>
<td>5.8</td>
<td>9.0</td>
</tr>
<tr>
<td>s.c. IFNβ-1a</td>
<td>25.0</td>
<td>37.8</td>
<td>43.3</td>
<td>15.6</td>
<td>33.6</td>
</tr>
<tr>
<td>s.c. IFNβ-1b</td>
<td>63.5</td>
<td>40.6</td>
<td>42.3</td>
<td>60.1</td>
<td>49.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th><strong>DRB1*04 positive</strong></th>
<th><strong>DRB1*04 negative</strong></th>
<th><strong>DRB1*15 positive</strong></th>
<th><strong>DRB1*15 negative</strong></th>
<th>Overall frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.m. IFNβ-1a</td>
<td>3.6</td>
<td>4.0</td>
<td>5.8</td>
<td>1.4</td>
<td>3.9</td>
</tr>
<tr>
<td>s.c. IFNβ-1a</td>
<td>14.9</td>
<td>21.2</td>
<td>27.7</td>
<td>4.5</td>
<td>19.0</td>
</tr>
<tr>
<td>s.c. IFNβ-1b</td>
<td>28.2</td>
<td>10.0</td>
<td>10.4</td>
<td>26.0</td>
<td>15.9</td>
</tr>
</tbody>
</table>

*Baseline risk* is the NAb registry based frequency of all IFNβ-treated MS patients who developed NAb and biologically relevant titers, i.e. a NAb titer >150 TRU/ml, for each treatment.

4.4.3 Discussion

HLA genotype influences the susceptibility to develop NAbs in the Swedish population, confirming that genetic factors predispose to the immunogenicity of IFNβ therapy [133-135], although the observed effects were modest compared to the influence of the respective IFNβ products themselves.

The identified associations to different HLA class II alleles are not unexpected given the central role of HLA class II molecules in peptide presentation to and activation of antigen-specific CD4+ T cells, which in turn provides necessary signals for persistent B-cell activation and induction of strong antibody responses [130]. Conversely, the overall association to HLA-B*07 is more difficult to interpret since there is no clear connection between HLA class I molecules and induction of humoral immunity. However, it is very likely that HLA-DRB1*15 and HLA-B*07 represents the same signal since HLA-B*07 is frequently present on the HLA-DRB1*15 haplotype because of high linkage disequilibrium between these allele groups. This is supported by
disappearance of the *HLA-B*07 association in the stratification for *HLA-DRB1*15 carriage in s.c. IFNβ-1a treated patients. We can, however, not decipher which of the alleles in the *HLA-B*07, *DRB1*15:01, *DQA1*01:02, *DQB1*06:02 haplotype that is responsible for this association.

Our results indicate that there seems to be different genetic factors determining the immunogenicity of the two different forms of human recombinant IFNβ molecules; *HLA-DRB1*15 for IFNβ-1a produced in mammalian cells, and *HLA-DRB1*04 for IFNβ-1b produced in *Escherichia coli*. Results from the predicted binding analysis imply that the peptide-binding motif of *HLA-DRB1*04:01 has better binding-affinity for IFNβ-1b peptides containing the cysteine-to-serine substitution at position 17. The results may also indicate that *HLA-DRB1*04:01 has high binding-affinity for IFNβ-1b peptides containing non-glycosylated asparagine at position 80. These product-specific HLA associations should be further investigated, preferentially by identifying peptides that bind to the HLA molecules *in vivo* in IFNβ-1a and IFNβ-1b treated individuals.

In this study, immunogenicity of IFNβ were detected using an *in vitro* neutralizing antibody assay and only patients who were positive for neutralizing anti-IFNβ antibodies were included, whereas in previous studies both binding and neutralizing anti-IFNβ antibodies were measured [133,134]. In addition to the different methods and definitions of antibody positivity, differences in HLA allele frequencies and distribution of treatments in the studied cohorts could lead to disparities in results between our study and previous studies by a German group [133,134].

This is the first study to describe associations to distinct HLA alleles for the two types of IFNβ molecules and it is possible that our finding occurred by chance, thus this finding needs to be confirmed in other studies with larger number of patients.
4.5 PAPER V

At the time of this study, in September 2011, almost 1600 natalizumab-treated MS patients had been routinely assayed for the presence of anti-drug antibodies against natalizumab in the NAb laboratory at Karolinska Institutet, as part of a post-marketing surveillance study of natalizumab treatment in Sweden. Since this is one of the largest single-center cohorts of natalizumab-treated MS patients being analyzed for development of anti-natalizumab antibodies, one objective of this study was to assess the prevalence of anti-natalizumab antibodies in a nation-wide clinical-based setting.

4.5.1 Prevalence of anti-natalizumab antibodies

Screening results from the 1391 included patients revealed that 0.9% of the patients (n=12) had a pre-treatment sample that was classified as antibody positive, although the reactivity against natalizumab was low compared to other positive samples and disappeared in nine out of twelve patients after treatment onset. This unexpected pre-treatment reactivity could be inflicted by the occurrence of rheumatoid factors (RFs) in these sera. RFs are low-affinity IgM antibodies directed against the Fc fragment of IgG and these autoantibodies, which are most often detected in individuals with suspected rheumatoid arthritis, are known to cause false positive results in immunogenicity assays [162]. Although all patients were negative for RFs the sera might contain other anti-immunoglobulin antibodies [163-165] leading to unspecific binding to natalizumab. Another potential reason for the pre-treatment reactivity, which cannot be excluded, is that these patients had received previous treatment with natalizumab without this being recorded on the referral forms or in the SMS registry.

After exclusion of patients with uncertain reactivity to natalizumab a total of 57 patients were considered positive for anti-natalizumab antibodies, giving an overall seroprevalence of 4.1% (57 out of 1379 patients). As previously observed, we found that antibody responses to natalizumab develop early after treatment onset [139,166]. In 96% of the positive patients, anti-natalizumab antibodies were detected in the first collected sample, after median treatment duration of three months (range 1-12 months). Except for the significantly shorter treatment duration at first sampling in the antibody positive group compared to the antibody negative group (3 months vs. 5 months) no other patient characteristic factors were observed to differ between the groups.

Further, sub-classification of positive patients revealed that 19 patients were persistently positive and 20 patients were transiently positive for anti-natalizumab antibodies. Since one third of the patients lacked a confirmatory follow-up sample, they were considered unconfirmed positive, and these 18 patients were not included in the subsequent analyses described below.

4.5.2 Prediction of persistent antibody response

The antibody response to natalizumab was further examined, with the aim to search for differences between transiently and persistently positive patients that could make it possible to predict if a patient is likely to become persistently positive or not.

Although the isotype and subtype specific analyses of the first positive sample from all transiently and persistently positive patients indicated that levels of anti-natalizumab IgM, IgG and IgG4 antibodies were significantly different between the groups, none of these antibody isotypes were specific or sensitive enough to be predictors for antibody status outcome. Instead we found that results from the standardized ELISA used in
routine screening could be used for distinguishing the groups from each other, since persistently positive patients had significantly higher total anti-natalizumab antibody levels compared to transiently positive patients. By calculating the ratio between the OD values from detection and competition wells, thus correcting for any non-specific reactivity of each sample, we identified an OD ratio of 40 to be a good cut-point for discriminating between the groups. Thus, by applying this calculation and using the established cut-off point we could predict the outcome, i.e. transient or persistent antibody status, in 80% of the cases only by using the first positive test results obtained from the screening ELISA.

In addition, when using an OD ratio of 40 as a cut-off point for the screening results from patients with unconfirmed positivity, 12 of the 18 patients had an OD ratio above 40 and were predicted to become persistently antibody positive. If correctly predicted, one might expect the overall frequency of persistently positive patients to be slightly higher than 1.4% and closer to 2% (Figure 5).

**Figure 5. Total antibody response of the anti-natalizumab antibodies.** The first positive samples after treatment start from transiently \((n=20)\), persistently \((n=19)\) and unconfirmed \((n=18)\) antibody-positive patients. Samples with an antibody concentration \(\geq 3\) μg/ml are shown as crossed shapes. Samples with an antibody concentration of 0.5-3 μg/ml are shown as filled shapes. The grey line indicates the predictive value for antibody persistence. The dotted line indicates the cut-off point for positivity.

### 4.5.3 Possible limitations of the study

We found that the overall occurrence of anti-natalizumab antibodies was 4.1%, which is slightly lower but still in accordance with what others have shown. Anti-drug antibodies have previously been detected in 4.5-14.1% of natalizumab-treated MS patients, of whom 1-4.7% had transiently occurring antibodies and 3.5-9.4% had persistently occurring antibodies [87,138-140,167]. Common for these studies, including our study is that the same standardized bridging ELISA method, developed by BiogenIdec, was used for screening of samples. Based on these results, the risk of developing ADA against natalizumab appeared to be a minor problem compared to other biopharmaceuticals.

However, the immunogenicity of natalizumab might be significantly higher than previously thought. In a paper published only weeks after our work was accepted, the authors could show that by using a radioimmunoassay (RIA) for antibody detection,
58% of patients were positive for anti-natalizumab antibodies at least once during the study [168]. This variation between studies is most likely the result of different methodologies used for assaying immunogenicity, as RIA methods are known to be more sensitive compared to ELISAs. The RIA method has also been shown to perform better at detecting anti-drug antibodies when free drug is present in the serum, since the bridging ELISA is more sensitive to drug interference [169]. Even so, both assays lack the ability to detect antibodies that have formed immune complexes with soluble natalizumab. Thus, it is likely that the proportion of antibody positive patients is largely underestimated in all of the studies performed so far.

One way of circumventing the problems with assay variability and immune complex formation is to measure serum natalizumab concentrations in order to predict treatment response in individual patients, since low serum natalizumab concentrations, below 1.0 µg/ml, seems to predict lack of treatment efficacy [168].

Although the ELISA may lack sensitivity to detect low levels of antibodies, with the possible consequence that we have underestimated the proportion of antibody-positive patients in our study, our results are still of clinical relevance. Since the cut-off point for positivity in the assay has been shown to correlate with the biological effect on serum levels of natalizumab [138], we are likely to detect those patients who develop antibody levels high enough to have a significant negative impact on the treatment effect.

4.5.4 Clinical relevance of findings

This study shows that by performing a single analysis using a screening ELISA, we can not only identify patients who are positive for anti-natalizumab antibodies but also determine whether antibody levels in positive samples are high enough to indicate a persistent immune response against natalizumab.

The diagnostic accuracy to predict persistence was slightly lower with our approach compared to the titration ELISA method described by Jensen et al. [167] (80% versus 90%). However, since our method requires no additional analyses of positive samples it is less time consuming and less costly, which is advantageous for routine analysis. Furthermore, our results are based on a standardized protocol that is proven to give reproducible results.

Previous recommendations for the re-monitoring of positive patients have been that everyone should be re-tested after at least six weeks. With support from the results of this study, we advise that positive patients with high antibody levels (OD ratio> 40) should be re-tested after four to eight weeks to confirm high antibody levels. In positive patients with lower titers (OD ratio <40), re-testing is advised after an extended time of three to six months. Meanwhile, treatment should be continued, unless hypersensitivity, other adverse events or treatment failure is suspected that require treatment discontinuation.

Based on these results, we can improve the monitoring of patients developing antibodies against natalizumab, which will assist clinicians when making decisions on whether a patient should discontinue treatment or not.
5 CONCLUDING REMARKS

Development of new recombinant human protein therapeutics for the treatment of a wide range of disorders such as MS and other chronic inflammatory diseases is increasing, making it even more important to prevent the development of ADAs. Identification of product-related factors that contribute to drug immunogenicity such as protein modifications, aggregate formation, and drug delivery regimes will hopefully result in less immunogenic treatments in the coming years.

Although the variation in NAb frequency between IFNβ preparations likely depends on differences in for example, frequency and site of injection, molecular and structural modifications and product purity, these differences do not entirely explain why most patients are immune tolerant to IFNβ. The results from study III and IV, imply that there may be shared immunological and genetic risk factors for the two IFNβ-1a preparations containing identical molecules, whereas the shared drug delivery site most likely contribute to the significantly higher immunogenicity of the two subcutaneously injected preparations IFNβ-1a and IFNβ-1b.

So far, only HLA has been shown to determine the immunogenicity of IFNβ, however, other genetic factors than HLA might influence the breakage of tolerance to IFNβ therapy. This is supported by a study by Weber et al. who identified one non-HLA SNP to be significantly associated with the anti-IFNβ antibody titers in MS patients treated with IFNβ. In addition, genetic polymorphisms in the genes encoding IL-10 and TNF-α have shown association to the development of anti-factor VIII antibodies, i.e. inhibitors, in hemophilia patients [170-172]. The functional analyses of these polymorphisms revealed that inhibitor development was more frequent in patients with production of high-TNF-α/high-IL-10 levels [170]. A recently published study describe that there are genome wide differences in gene expression between HLA-DRB1*15:01 positive and HLA-DRB1*15:01 negative MS patients [173]. Among the ~1200 exons identified to be differentially expressed between these two MS groups were genes known to be involved in distinct immune signaling pathways such as IL-4 and IL-17 signaling. Whether these differences between HLA-DRB1*15:01 carriers and non-carrier could influence our findings in study IV would be interesting to investigate.

Cigarette smoking has been confirmed to be an environmental/life-style factor that increases the susceptibility to develop MS [54-56], and there is also a possible correlation between smoking and disease progression [174,175]. Interestingly; smoking has also been shown to influence the risk for MS patients to develop ADA against both IFNβ [136,137] and natalizumab [176]. These findings suggest that the lung is an immune-competent organ, which not only impact disease susceptibility but also influences the response to therapy. Thus, there is a potential benefit of smoking cessation in MS patients planning to initiate treatment with BPs. The importance of environmental/life-style factors for ADA development has only begun to be explored, and it would also be of interest to study whether the immune modulating effects of sunlight and/or vitamin D [177] can have a protective effect on the development of ADA responses.

NAb against IFNβ can persist for several years after stopping treatment [178,179]. Furthermore, NAb against recombinant IFNβ therapy cross-react with and neutralize the effect of endogenously produced IFNβ in vitro [180]. Thus, in patients with persistent NAb the endogenous IFNβ response may be hampered, possibly making
these patients more susceptible to viral infections. Although this may not lead to severe consequences in MS patients, the persistence of ADA in patients receiving replacement therapy with factor VIII or hormones could be life-threatening. There is thus a need to find ways to efficiently deplete the ADA response once they have developed to be able to reduce the consequences for treated patients. This could be done with immune tolerance induction (ITI) therapy or with therapies targeting B cell responses, e.g. rituximab. These are alternatives that need to be investigated further.

In conclusion, since the introduction of BPs there have been major improvements in the treatments of several different diseases, including MS. To make these treatments more efficient and safer for the patients it is important that we increase our understanding of the immunological mechanisms leading to ADA responses, and how genetic and environmental factors may influence the immunogenicity of recombinant human proteins. By taking the development of ADAs into consideration and by including ADA analysis in routine practice the ineffective treatment of patients can be avoided, hopefully leading to better patient care and lower medical costs in the future.
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