Natalizumab

The road towards personalized medicine
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Chapter 1

General introduction
General introduction

Multiple Sclerosis (MS) is an inflammatory immune mediated demyelinating disease of the central nervous system that was first described in detail in 1868 by Jean-Marie Charcot. It is nowadays the most common chronic disabling neurological disease in young adults. MS is characterized by attacks of neurological symptoms and increasing disability. It is generally thought that MS has a multifactorial origin: a combination of genetic susceptibility and environmental factors. Especially, the last years, a lot has been changed in the treatment of relapsing remitting multiple sclerosis (RRMS). Many new, sometimes even more effective treatments became available, but with these new treatments, also more serious side effects were seen. Natalizumab is a highly effective treatment, but associated with an increased risk to develop progressive multifocal leukoencephalopathy (PML). PML is an opportunistic infection of the central nervous system (CNS), which is caused by reactivation and replication of the JC virus (JCV) and is potentially life threatening. The effectiveness and the side effects have to be balanced and will be different in every patient. With this thesis, we hope that the road towards personalized medicine in the treatment with natalizumab comes closer.

Pathogenesis

Traditionally, MS is thought to be an autoimmune disorder of the central nervous system involving inflammation, demyelination and axonal degeneration, but the exact etiology of MS is still unknown. Focal lesions in the white matter of the brain and spinal cord characterize MS. Nowadays we know that also the grey matter is involved in this disease. These focal lesions are thought to be a result of demyelination by inflammation. The generally accepted hypothesis is that the inflammation results from an inappropriate response of auto reactive T cells against myelin or oligodendrocytes. Only a small part of the new inflammatory lesions will give rise to neurological symptoms, many new lesions are thus asymptomatic. Besides the focal lesions, loss of brain volume, which can be measured by magnetic resonance imaging (MRI) and is reflecting axonal loss, is seen. Axonal loss is described at all clinical stages of the disease course and seems to occur
independent of the inflammatory processes. Axonal damage seems to be permanent and probably contributes to the irreversible disability in MS patients, particularly in the progressive forms.

**Epidemiology**

The prevalence of MS is about 100-200 per 100,000 people in Western European countries and increases with a larger distance of the equator. In the Netherlands approximately 17,000 people have MS. The average age of onset is 30 years and females are at least twice as often affected as men.

**Clinical manifestations**

Based on the clinical course, different phenotypes have been described (Figure 1). The most common form is relapsing remitting (RR) MS, which affects around 85% of the MS patients. It is characterized by acute or subacute episodes of focal neurological deficits affecting the optic nerve, cerebral hemispheres, brainstem, cerebellum or spinal cord. The first episode of neurological deficits is defined as clinically isolated syndrome (CIS). About 30-70% of the CIS patients will develop MS with a RRMS course. Focal neurological deficits can be monocular visual loss, sensory complaints, a feeling of electric discharges along the spine on head flexion (Lhermitte’s sign), diplopia, dizziness, bladder dysfunction or walking problems. These periods of (sub)acute neurological symptoms can resolve with partial or complete remission. Around 65% of these patients eventually develop, after a phase of relapses and remissions, a slow increase of neurological disability, so called secondary progressive (SP) MS. The remaining 15% of the patients have progressive disability starting from the onset of the disease, which is called primary progressive (PP) MS. PPMS is more common in males and manifest at a later age. Superimposed relapses are infrequently seen in PPMS patients. The description of the clinical course of multiple sclerosis is revised in 2013 by Lublin et al. We now distinguish two important modifiers of the disease course. The first one is an evaluation of disease activity, which is defined clinically as occurrence of relapses or
radiologically as lesion activity (gadolinium positive lesions and/or new/enlarging T2 lesions). So, by example, a patient without a new relapse in the past year, but with a new gadolinium-enhancing lesion on the MRI scan will be considered RR-active. The second modifier is progression of disability, which is defined as whether or not there is clinical evidence of disease progression, independent of relapses, over a given period of time (at least annually) in patients with SPMS or PPSMS. A patient known with PPMS and over the last year no relapse, but with clinical progression which is not recovering will be considered as PP – not active but with progression\(^9\). The course and prognosis of MS is highly variable in patients with MS\(^{10}\).

![Graphs of RR, SP, and PP disease courses](image)

Figure 1. Schematic illustration of the clinical disease course of the different subtypes of MS. The X-as is representing time and the Y-as neurological disability. RR = relapsing remitting, SP = secondary progressive, PP = primary progressive.

Besides the diagnosis MS, a spectrum of inflammatory demyelinating diseases exist, including myelitis transversa (MT), neuromyelitis optica (NMO, also called Devic’s disease), acute disseminated encephalomyelitis (ADEM), Marburg’s disease and Balo’s concentric sclerose\(^{12}\).

**DIAGNOSIS**
Originally, the diagnosis of MS was based on the occurrence of two or more clinical episodes of neurological deficits, which last more than 24 hours and at least 30 days apart (dissemination in time) and can only be explained by demyelination at different sites in the central nervous system (CNS) (dissemination in space). The first diagnostic criteria were developed in 1983 and called the Poser criteria\textsuperscript{13}. Results from evoked potentials and cerebrospinal fluid analysis (oligoclonal bands or increased IgG index) were incorporated in these criteria. Since 2001, MRI is adapted as well (McDonald criteria)\textsuperscript{14}. The diagnostic criteria for MS have been changed over the years\textsuperscript{15,16}. Nowadays, dissemination in time and space is based on or clinical findings or on a combination of clinical and MRI findings (Table 1)\textsuperscript{16}. The use of the MRI for demonstration of dissemination in time and space has been simplified. In some patients the diagnosis RRMS can now be made by only one clinical episode and one MRI scan of the central nervous system. These modifications made it possible, with maintenance of the diagnostic sensitivity and specificity, to diagnose MS earlier\textsuperscript{16}.
### Table 1: The revised McDonald 2010 criteria for diagnosis of MS\textsuperscript{16}.

<table>
<thead>
<tr>
<th>Clinical Presentation</th>
<th>Additional Data Needed for MS Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2 attacks\textsuperscript{a}; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack\textsuperscript{b}</td>
<td>None\textsuperscript{c}</td>
</tr>
<tr>
<td>≥2 attacks\textsuperscript{a}; objective clinical evidence of 1 lesion</td>
<td>Dissemination in space, demonstrated by:</td>
</tr>
<tr>
<td></td>
<td>≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)\textsuperscript{d}; or Await a further clinical attack\textsuperscript{a} implicating a different CNS site</td>
</tr>
<tr>
<td>1 attack\textsuperscript{a}; objective clinical evidence of ≥2 lesions</td>
<td>Dissemination in time, demonstrated by:</td>
</tr>
<tr>
<td></td>
<td>Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack\textsuperscript{a}</td>
</tr>
<tr>
<td>1 attack\textsuperscript{a}; objective clinical evidence of 1 lesion (clinically isolated syndrome)</td>
<td>Dissemination in space and time, demonstrated by:</td>
</tr>
<tr>
<td></td>
<td>For DIS:</td>
</tr>
<tr>
<td></td>
<td>≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)\textsuperscript{d}; or Await a second clinical attack\textsuperscript{a} implicating a different CNS site; and For DIF:</td>
</tr>
<tr>
<td></td>
<td>Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack\textsuperscript{a}</td>
</tr>
<tr>
<td>Insidious neurological progression suggestive of MS (PPMS)</td>
<td>1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria\textsuperscript{d};</td>
</tr>
<tr>
<td></td>
<td>1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions</td>
</tr>
<tr>
<td></td>
<td>2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord</td>
</tr>
<tr>
<td></td>
<td>3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)</td>
</tr>
</tbody>
</table>

### Treatment

There are three types of treatment in MS: Treatment of (sub) acute new symptoms (also called: relapse, exacerbation or schub), treatment to reduce the frequency and severity of relapses and symptomatic treatment. There is no effective treatment for the progression of the disease, so for SPMS and PPMS yet.

An invalidating relapse can be treated with corticosteroids. Corticosteroids have no proven effect on the relapse outcome or long-term disease progression, but can shorten
and accelerate the recovery of the relapse. There is no consensus about the optimal form, dosage, route of administration or duration of corticosteroid therapy\textsuperscript{17}. Nowadays, to reduce the frequency and severity of relapses, a variety of disease modifying treatments (DMT’s) for RRMS have been registered by the European Medicines Agency (EMA). In 1995, the first DMT approved for the diagnosis MS was interferon (IFN) beta-1b (Betaseron®). Over the years, also interferon beta-1a (Avonex® and Rebif®) and glatiramer acetate (GA) (Copaxone®) were approved for the treatment of RRMS. The most noticeable result of all trials was a reduction in the frequency (approximately 30%) and severity of the relapses\textsuperscript{18,19,20,21}. Also, multiple studies found a reduction of gadolinium (Gd+) enhancing lesions and/or new T2 lesions\textsuperscript{22,23,24}. IFN-beta and GA are registered as first-line therapies in MS and have nowadays a well-known favourably long-term safety profile. All these abovementioned therapies are injectables, which give rise to some compliance issues\textsuperscript{25}. Mitoxantrone, nowadays barely used in the Netherlands because of its severe adverse events, is a strong immunosuppressant, which is administered intravenously with a 3 months interval. It reduces the ARR by 60% and the disability progression by 64\%\textsuperscript{26}. The most important adverse events are cardiotoxicity and therapy-related acute leukaemia (mortality of about 40% occurs in 1% of the patients), often several years after discontinuation of the mitoxantrone. Besides, amenorrhoea was seen in more than 20% of the fertile women\textsuperscript{27,28}. Natalizumab, a very effective, second-line therapy for RRMS was approved in 2006. It is given by infusion, 300 mg every four weeks. Natalizumab will be extensively discussed later this chapter. Since 2010, there is a fast increase in oral treatments for RRMS. The first one, fingolimod (Gilenya®) modulates the sphingosine-1-phosphate receptors and thereby inhibits the egress of lymphocytes from the secondary lymphoid tissues, preventing auto-reactive lymphocytes to migrate into the circulation and the central nervous system (CNS)\textsuperscript{29}. Three phase 3 trials showed a significant effect on annualized relapse rate (ARR). In the FREEDOMS and FREEDOMS II trial, a reduction of 48-54% was seen in ARR in patients using fingolimod compared to placebo\textsuperscript{30,31}. In the TRANSFORMS trial, fingolimod showed a significant lower annualized relapse rate in both dosage groups compared to placebo. In the fingolimod patients receiving 1.5 mg, the annualized relapse rate was
0.20, in the patient group using 0.5 mg this was 0.16 compared to 0.33 in the interferon group\textsuperscript{32}. In the 1-year extension of the FREEDOMS trial and the up to 4.5 years extension of the TRANSFORMS trial, a persistent reduction in ARR was found\textsuperscript{33,34}. Fingolimod also showed efficacy on the secondary MRI outcome parameters, i.e. Gd+ lesions, new or enlarged T2 lesions, and brain volume measures\textsuperscript{30,32}. Only in the FREEDOMS trial, also a reduced disability progression was seen compared to placebo at 24 months\textsuperscript{30}. Because of specific safety issues, herpes virus dissemination, tumour development, potential cardiac side effects (bradyarrhythmia and atrioventricular block) and unknown long-term safety data, fingolimod 0.5 mg is in 2011 registered by the EMA as second-line treatment for patients with highly active RRMS despite treatment with INF-beta or GA, or RRMS patients with rapidly evolving severe disease activity. Recently it became clear that there is a risk to develop progressive multifocal leukoencephalopathy (PML). Until January 2016, five cases of PML in MS patients treated with fingolimod and not attributable to natalizumab were reported. The risk is now estimated at 0.037/1.000 patients\textsuperscript{35}. PML is a potential life threatening disease, caused by reactivation of the John Cunningham (JC)-virus. Later in this chapter, PML will be more extensively discussed.

Since March 2013, two new oral therapies were registered as first-line therapy for the diagnosis RRMS, namely teriflunomide (Aubagio\textsuperscript{®}) and dimethyl fumarate (DMF) (Tecfidera\textsuperscript{®}).

Teriflunomide reduces the proliferation of lymphocytes by blocking the mitochondrial enzyme dihydroorotate dehydrogenase. Two phase 3 trials, TEMSO and TOWER, showed a significant reduction in ARR in patient groups using teriflunomide compared to placebo\textsuperscript{36,37}. In the TEMSO trial an ARR of 0.37 was found for teriflunomide for the 7 and 14 mg dosage compared to 0.54 for placebo, which means a relative risk reduction of 31.2% and 31.5% for the 7 and 14 mg fingolimod, respectively\textsuperscript{36}. In the TOWER study, the annualized relapse rate was higher in patients assigned to placebo (0.50 [95% CI 0.43–0.58]) than in those assigned to teriflunomide 14 mg (0.32 [0.27–0.38]) or teriflunomide 7 mg (0.39 [0.33–0.46]). This effect of teriflunomide on the ARR is in the same range as IFN-beta and GA (31% reduction of ARR compared to placebo)\textsuperscript{36,37}. In both studies, only teriflunomide 14 mg reduced the risk of sustained accumulation of disability compared to placebo, no effect for teriflunomide 7 mg was seen. Teriflunomide also showed to have a significant effect on radiological parameters, i.e. a significant
reduction of Gd+ lesions and total lesion volume. There are no known serious adverse effects\textsuperscript{36,37}.

The other new oral medication is dimethyl fumarate (DMF), also known as BG-12. DMF and its active metabolite monomethyl fumarate activates the nuclear factor E2-related factor-2 pathway (Nrf2), which protects against oxidative-stress-related neuronal death and damage to myelin in the CNS. In two phase 3 trials, CONFIRM and DEFINE, a significant reduction of the ARR was seen compared to placebo\textsuperscript{38,39}. In the CONFIRM study, the relative reduction of annualized relapse rate was 44% with the twice a day DMF, 51% with the three times a day DMF and 29% in the patients using GA compared to placebo. In the DEFINE study, the relative reduction was 53% in the twice a day DMF and 48% in the three times a day DMF compared to placebo. There was no significant reduction in disability progression seen comparing DMF to placebo in the CONFIRM study. However, in the DEFINE study, there was a significant relative reduction in disability progression of 38% in the twice a day DMF and 34% in the three times a day DMF patient group compared to placebo. In the CONFIRM study, DMF significantly reduced the number of new or enlarging T2 lesions and new T1-weighted hypointense lesions compared to placebo. It also significantly reduced the number of new or enlarging T2-weighted hyperintense lesions in the DEFINE study. Also, in this study, the number of gadolinium-enhancing lesions was significantly reduced compared to placebo. Gastro-intestinal events and flushing were the most common adverse events.

There are now four MS patients treated with Tecfidera®; who developed progressive multifocal leucoencephalopathy (PML)\textsuperscript{40,41}. Three patients developed PML in the setting of severe lymphopenia (<0.5x10\textsuperscript{9}/L) and one patient in the setting of moderate lymphopenia (approximately 0.6x10\textsuperscript{9}/L persisting for >6 months. Rare cases of PML have also been reported in patients who developed lymphopenia during treatment with compound dimethyl fumarate (Fumaderm®) for psoriasis\textsuperscript{42,43,44,45,46}. In the first described cases, there was severe lymphocytopenia\textsuperscript{42,43}. In later cases dimethyl fumarate-associated PML was also described in patients with less severely reduced lymphocytes\textsuperscript{44,45,46}. Nieuwkamp et al. described a patient with no prior immunosuppressive medication in whom the lowest lymphocyte count was 792 cells per cubic millimeter\textsuperscript{45}.

The last therapy now registered in Europe for RRMS is alemtuzumab (September 2013). Alemtuzumab is a humanized monoclonal antibody directed against CD52 to deplete
circulating T- and B-lymphocytes. This is followed by a distinctive pattern of T- and B-cell repopulation, which results in an alteration of the balance of the immune system. This medication is given intravenously once daily for 5 days the first time and for 3 days a year later. There was a clear reduction of relapses compared to subcutaneous IFN-beta 1a (about 50% more reduction)\textsuperscript{47,48}. In these studies there was also an effect on the radiological parameters, as Gd+ lesions and new or enlarged T2-lesions, and in relative reduction in disability progression\textsuperscript{47,48}. The down side of this very effective therapy is that alemtuzumab frequently cause infusion reactions, infections and at least 30% of the patients develop autoimmune thyroid conditions. Also, idiopathic thrombocytopenia has been detected in 1-3% of the patients and a few patients have developed Goodpasture’s syndrome (renal failure)\textsuperscript{49}.

For some problems in MS, symptomatic (farmacological) therapies exist to reduce the complaints. The most important symptomatic problems are cognitive dysfunction, depression, fatigue, spasticity, gait impairment, sphincter dysfunction (mostly bladder dysfunction) and sexual dysfunction. For cognitive dysfunction in MS, there are no proven farmacological therapies\textsuperscript{50}. The management of symptoms of a depression is the same as in patients without MS. Fatigue is a very common complaint in MS and may for example be treated with amantadine or methylphenidate, but overall no major effect of these medications have been shown in studies\textsuperscript{51,52}. For spasticy oral baclofen, tizanidine and dantrolene are proven to be effective\textsuperscript{53}. Prolonged-release fampridine leads to a meaningful effect on the walking ability in a subgroup of MS patients\textsuperscript{54,55,56,57}. Since April 1\textsuperscript{st} 2016, Fampyr\textsuperscript{a}® (prolonged-release fampridine) is temporary compensated in the Netherlands by the insurance company for the indication of walking disability in MS patients. Oxybutin and tolterodine are first-line therapies to treat bladder dysfunction\textsuperscript{58}. OnabotulinumtoxinA injections into the detrusor muscle, used in case of intolerance or refractory detrusor overactivity on first-line medication, are also proven to be effective\textsuperscript{59}. With the introduction of the pro-erectile oral medications as sildafenil, the treatment options for sexual dysfunction have extended.

**Natalizumab**

Because natalizumab is the subject of this thesis, we will more extensively discuss this treatment here in this separate chapter.
Natalizumab (Tysabri®, Biogen Idec) is a humanized monoclonal antibody binding the α4 chain of α4β1 and α4β7 integrin on leukocytes. Therewith, natalizumab blocks the adhesion of leukocytes to the vascular endothelium (VCAM-1 and MAdCAM-1) and so prevents the extravasation of leukocytes into the CNS in patients with MS and into the intestinal mucosa in patients with Crohn's disease. By preventing T lymphocytes, B lymphocytes and plasma cells to migrate into the CNS, inflammatory reactions in patients with RRMS will be suppressed.

Two phase 3 trials were done before natalizumab was approved in the Netherlands in 2006 for treatment of RRMS. In the AFFIRM trial, natalizumab was tested as a monotherapy and was compared to placebo. In the SENTINEL trial patients were receiving natalizumab or placebo, but all patients continued interferon beta-1a as well. Natalizumab showed a 68% reduction in relapse rate over 2 year compared to placebo. Besides, a significant reduction of Gd+ lesions (92%), new or enlarged T2 lesions (83%) and disability progression (54%) was seen. In these studies, 9% of the patients developed antibodies against natalizumab. These antibodies were persistent in 6% and transient in 3% of the patients. Persistent antibody positive patients had less clinical efficacy and more infusion reactions.

The most common adverse events are fatigue, infections, rash with itchiness, headache, dizziness and nausea. A less frequent (1:1000) adverse event is an severe allergic reaction. The most serious complication of natalizumab treatment nowadays is the occurrence of progressive multifocal leukoencephalopathy (PML). PML is an opportunistic infection caused by reactivation and replication of the JC virus (JCV). The SENTINEL trial was stopped in February 2005, about one month early because two patients in the natalizumab arm developed PML. Besides, a patient with Crohn’s disease treated with natalizumab developed PML as well. In this last patient, a new and increasing viral load of JCV in serum was seen, which was absent before the initiation of natalizumab therapy, and supported the relationship between natalizumab therapy and the development of PML. The role of the concomitant administration of interferon beta-1a was at that moment unclear. In mid-2006, natalizumab was as monotherapy reintroduced on the market. Since then, worldwide, 617 confirmed cases of PML in natalizumab-treated RRMS patients have been reported.

**Progressive multifocal leukoencephalopathy**
PML is a severe demyelinating disease of the CNS, caused by the polyomavirus JC (JCV), and was initially described in 1958 in two patients with chronic lymphocytic leukaemia and one patient with Hodgkin lymphoma. The name JC virus was derived from the initials of the first patient with PML (John Cunningham) whose brain tissue in 1971 was used for culture and isolation of the virus. The primary infection with JCV usually occurs in childhood, is assumed to be in the tonsillar stromal cells, and is in general asymptomatic. After the primary infection, the virus remains latent in the kidneys, bone marrow and lymphoid tissue. Alteration of the normal immune function can result in reactivation of the virus, causing infection of oligodendrocytes, astrocytes, and progenitor cells in the brain by peripheral B-lymphocytes.

Until 1980, PML remained a rather rare disease; occurring apart from lymphoproliferative and myeloproliferative diseases, in patients with malignancies, granulomatous and inflammatory disorders, as well as in organ transplant receivers. In the 1980s, with the beginning of the human immunodeficiency virus (HIV) epidemic, the incidence of PML was extremely increased. PML became soon a well-known opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS).

The overall incidence of natalizumab-associated PML worldwide is now 4.15:1000 patients (95% CI 3.83 to 4.48 per 1000 patients). Risk factors for the development of PML are anti-JCV antibodies (especially a anti-JCV index >1.5), long duration (>2 years) of natalizumab treatment and previous immunosuppressive medication. In the patients with positive anti-JCV antibody status, prior immunosuppressive medication and a treatment duration Of 25-48 months, the incidence is up to 11:1000 patients.

Classic PML is, as the name implies, a progressive, multifocal disease involving the white matter. Nowadays, we know that it can also present as a single lesion, can be non-progressive, can involve the grey matter and can be associated with inflammation. Clinically, PML is characterized by subacute neurologic deficits, including cognitive impairment, motor deficits and visual symptoms. Radiologically, subcortical multifocal white matter lesions are the classic MRI feature of PML. Besides, Gd+ enhancement is frequently seen and can help in diagnosing PML. Nowadays, PML is more frequently diagnosed in natalizumab-treated MS patients by MRI in a clinical asymptomatic stage of the disease. The MRI in asymptomatic PML shows frequently a quite localized
disease, with often lesions in the frontal lobes, the cortical grey matter and adjacent juxtacortical white matter\textsuperscript{87}. Of the natalizumab-associated PML patients who had at least a follow-up of 6 months, 77\% survived PML with varying levels of disability\textsuperscript{68}. In case of early diagnosis, the prognosis of PML seems better\textsuperscript{84,86,88,89}. If PML is suspected, natalizumab treatment should be stopped immediately and active washout of the natalizumab by plasmapheresis (PLEX) or immunoabsorption (IA) to restore the immune surveillance is treatment of first choice\textsuperscript{90,91,92}. Mefloquine, an antimalaria agent, might be effective in PML based on a in vitro study in which the activity of JC virus was studied on a human glial cell line\textsuperscript{93}. Also mirtazapine, an inhibitor of the 5-HT2a-receptor, has shown to inhibit the infection of JC virus on a human astroglial cell line in vitro\textsuperscript{94}. In clinical practice, there is no proof for these treatments yet\textsuperscript{92}.

**Immune reconstitution inflammatory syndrome (IRIS)**

Within days to weeks after the PLEX/IA, patients will experience an increase of clinical symptoms. The MRI scan frequently shows enlargement of lesions with Gd+ enhancement. This syndrome is called immune reconstitution inflammatory syndrome (IRIS) and is caused by the reconstitution of the immune system by washing out the natalizumab from the body. In general IRIS is treated with corticosteroids. It is also suggested that without the use of glucocorticosteroids, maraviro could contribute to the initial prevention and the active treatment of IRIS (also in HIV-associated PML-IRIS) by selectively reducing the CCR5+ leukocytes, which seems to be the mediators of IRIS, into the central nervous system (CNS)\textsuperscript{95,96,97}.
Aims and outline of this thesis

Natalizumab is, as has become clear from the preceding paragraphs, a very effective treatment for patients with RRMS. Unfortunately, with the potential side effect of PML. In this thesis we want to answer two important questions on the road towards more personalized medicine in the treatment of natalizumab. We first aim to find out which factors have a relevant influence on the effectiveness of the treatment of natalizumab. Secondly, we aim to discover the most important factors which are contributing to the development of PML. Ultimately, we hope this knowledge will contribute to a more individualized approach of natalizumab treatment in RRMS patients.

In chapter 2 we studied several factors that were thought to be of importance for differences in disease activity in natalizumab treated patients and after cessation of the drug. In chapter 2.1 we studied the clinical and radiological impact of the serum natalizumab concentrations and their relationship with anti-natalizumab antibodies. Initially, the hypothesis was that if you regularly interrupt the natalizumab treatment in MS patients, you will decrease the risk of developing PML, but evidence at that moment was lacking. So, in chapter 2.2 we observed a small group of patients who we precisely monitored clinically and radiologically to discover the consequences of a so called “natalizumab holiday”. Cessation of natalizumab is associated with recurrence of clinical and radiological disease activity, sometimes in a way it is even suggesting a so called rebound of disease activity. This re-activation of disease activity occurs most of the time between two and six months after cessation of the natalizumab therapy\textsuperscript{98,99}. Clearance of natalizumab has only been studied for relatively short periods of time\textsuperscript{90}. To get more insight in the clearance of natalizumab, we studied natalizumab levels for up to 260 days after discontinuation of the treatment (chapter 2.3).

In chapter 3 we focussed on serological biomarkers and their relevance in natalizumab-associated PML. JCV seropositivity is a risk factor for developing PML in natalizumab-treated MS patients. When JCV seronegative MS patients seroconvert, their risk of developing PML is increasing. In chapter 3.1 we investigated if there is more JCV seroconversion in natalizumab-treated MS patients than might be expected based on natural history data and published by Gorelik et al.\textsuperscript{100}. It has been suggested that there is a higher risk of developing PML in natalizumab-treated MS patients with high serum
anti-JCV antibody indexes in pre-PML samples, as well as that an increase of this index is seen preceding the development of PML\textsuperscript{101,102,103,77}. In these studies, very limited longitudinal samples were available, so in \textbf{chapter 3.2} we studied four natalizumab-treated MS patients who developed PML in whom extensive pre-PML samples were available to study if these suggestions could be confirmed.

The outcome of natalizumab-associated PML depends on restoration the immune function. To reach this, PLEX/IA can be given to accelerate the clearance of natalizumab from the body. The standard regime till now is to give these patients 5 sessions of PLEX/IA. However, there may be interindividual differences. In \textbf{chapter 3.3} we studied if serum natalizumab guided PLEX in PML patients would prevent unnecessary PLEX treatments in some patients and inadequate premature cessation in others.

In the summarizing discussion and future perspectives we will discuss how this knowledge may lead to a more individualized approach of natalizumab treatment in RRMS.
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Chapter 2

Natalizumab in the treatment of multiple sclerosis
Chapter 2.1

Clinical relevance of serum natalizumab concentration and anti-natalizumab antibodies in multiple sclerosis

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Abstract

**Background:** Antibodies against natalizumab have been found in 4.5-14.1% of natalizumab-treated multiple sclerosis (MS) patients. If antibodies persist, they are associated with an adverse effect on treatment response. However, it has proved to be difficult to standardize anti-drug antibody measurements.

**Objectives:** The purpose of this study was to evaluate the clinical and radiological impact of serum natalizumab concentrations and their relation with anti-natalizumab antibodies in MS patients.

**Methods:** In this prospective observational cohort study of 73 consecutive patients treated with natalizumab, we measured serum natalizumab levels and antibody titers before the start of natalizumab treatment, at week 12 and 24 and annually after natalizumab initiation. Antibodies against natalizumab were measured by radioimmunoassay and serum natalizumab concentrations using a newly developed enzyme linked immunosorbent assay (ELISA). Magnetic resonance imaging (MRI) scan and clinical evaluation were performed before the start of natalizumab treatment and subsequently every year.

**Results:** Antibodies were detected in 58% of the natalizumab-treated patients. All patients developed their antibodies before week 24. The large majority of these patients reverted to neutralizing antibody (Nab) negative status during follow-up. The presence of antibodies was inversely correlated with serum natalizumab concentration ($p<0.001$). Only high antibody titers are associated with very low or undetectable serum natalizumab concentration. Both high antibody titers and low serum natalizumab concentrations are associated with relapses and gadolinium-enhancing lesions on MRI.

**Conclusions:** Our data show that both low natalizumab serum concentration and high antibody titers are associated with a lack of efficacy of natalizumab. Measuring serum natalizumab, using a highly specific assay, might lead to more enhanced precision using natalizumab in individual patient.
Introduction

Natalizumab is a humanized recombinant monoclonal antibody directed against very late active antigen (VLA)-4. Natalizumab binds to the α4 chain of α4β1 integrin (VLA-4) and α4β7 integrin and blocks the migration of leucocytes across the blood-brain barrier into the central nervous system (CNS) and therewith suppresses the inflammatory reaction in patients with relapsing-remitting multiple sclerosis (RRMS).\(^1\,2\) In multiple phase III studies, natalizumab significantly reduced the annualized relapse rate and the sustained disability progression compared both to placebo and to interferon-beta (IFN-β). Furthermore, it reduced the number of gadolinium positive (Gd+) lesions as well as the number of new and enlarging T2-hyperintense lesions.\(^3\,4\) Due to the risk of progressive multifocal leucoencephalopathy, natalizumab is only recommended for patients with RRMS who have an inadequate response to first-line immunotherapy or have very active relapsing disease.

Nearly all protein therapeutic agents induce neutralizing antibodies (NAbs), which often reduce efficacy.\(^5\)-\(^10\) NAbs against natalizumab can develop early during treatment and have been found in 4.5-14.1% of natalizumab-treated multiple sclerosis (MS) patients, of whom 3.5-9.4% were persistently positive and 1-4.7% transiently positive.\(^11\)-\(^13\) Calabresi et al. found that persistent antibody positivity reduced serum natalizumab concentrations and had an adverse effect on treatment response and a higher incidence of infusion-related adverse events, including hypersensitivity reactions.\(^11\) Interestingly, a recent Danish study, using an extended enzyme linked immunosorbent assay (ELISA) method, suggested that after three months of natalizumab, patients who were persistently NAb positive had higher titers than transiently positive patients. Their findings suggest that testing at three months may be helpful in selecting patients who should discontinue natalizumab infusions.\(^14\)

Here, we investigated whether antibody formation leads to decreased levels of free circulating natalizumab and whether measuring serum natalizumab concentrations has clinical relevance in addition to measuring antibodies alone. We report a combined analysis of natalizumab concentrations, measured by means of a newly developed ELISA, and anti-natalizumab antibodies measured by a radioimmunoassay (RIA), in serum of well-monitored RRMS patients treated with natalizumab.
Methods

Patients and study design
A prospective observational cohort study was performed from March 2007 to March 2010 at the MS Centre of the VU Medical Centre in Amsterdam, the Netherlands. During that period, 73 consecutive patients with RRMS treated with natalizumab, and of whom at least one blood sample was available after starting the natalizumab treatment, were included. These patients either failed on standard immunotherapeutic agents (IFN-β or glatiramer acetate (GA)), or were unable to tolerate these drugs.

Blood samples were obtained before the start of natalizumab (i.e. baseline) and every 12 weeks thereafter, just before the infusion was given. The serum samples were stored at -80ºC until assayed at Landsteiner Laboratory Sanquin Research, in Amsterdam, the Netherlands. We measured serum natalizumab levels and antibody titers at baseline, 12 and 24 weeks, and annually after the natalizumab initiation. Magnetic resonance imaging (MRI) scans and clinical evaluation, including evaluation of relapses and Expanded Disability Status Scale (EDSS), were performed at baseline and subsequently every year. This study was approved by the local institutional board. Informed consent was obtained from all participants.

Measurement of serum natalizumab levels
Serum natalizumab levels were measured by developing a cross-linking assay, in which specific polyclonal rabbit anti-natalizumab F(ab)2 fragments are used as capture reagents and a mouse anti-IgG4 monoclonal antibody is used for detection as described elsewhere. The detection limit of the assay is about 0.01 µg/ml. Since the number of patients was relatively small and only a few had disease activity, formal Receiver Operating Characteristic (ROC) curves were not possible to determine the best cut-off point for the serum natalizumab concentration. We used the cut-off point described by Khatri et al., who described how desaturation of α4-integrin was observed to be less than 50% when natalizumab concentrations were below 1µg/ml. Patients were therefore categorized as having low natalizumab concentrations if the natalizumab concentration was below 1.0 µg/ml.

Measurement of antibodies against natalizumab
Anti-natalizumab antibodies were measured with a newly developed RIA essentially following the protocol described by Bartelds et al. Serum of patients was incubated with Protein A Sepharose for catching IgG from serum and 125I radioactive labelled F(ab)2 fragments of natalizumab to detect the IgG anti-natalizumab antibodies. After overnight incubation, unbound radiolabel was washed out and Sepharose-bound radioactivity was measured and converted into arbitrary units (AU) by comparison to a reference serum. By adding F(ab’)2 fragments of polyclonal multidonor IgG (Freeze buffer, Sanquin) the assay would only detect anti-idiotype antibodies. Patients were defined as antibody negative if the anti-natalizumab antibody concentration was <12 AU/ml and antibody positive if the antibody concentration was ≥ 12 AU/ml. This cut-off is based on the mean +3 SD measured in 100 healthy donors (data not shown). Based on analyses of antibody formation to adalimumab we distinguished between low antibody (≥12-100 AU/ml) and high-antibody concentrations (>100 AU/ml).

**Measurement of radiological and clinical data**

MRI scans were performed on a 1.5 Tesla (Siemens AG, Erlanger, Germany) scanner with 8ch head coil, using standard 2D conventional or fast spin echo proton density (PD) and T2-weighted images (repetition time 2700 ms, echo time 45 and 90 ms) with slice thickness of 5 mm, a maximum gap between slices of 0.5 mm, and an in plane solution of 1 x 1 mm². An independent radiologist, who was blinded for clinical and laboratory data, rated the development of new or enlarged T2-weighted lesions and Gd+ T1-weighted lesions on brain MRI compared to baseline.

Relapses and EDSS were scored blind for MRI, serum natalizumab and antibody concentrations. Relapses were defined as the appearance of a new symptom or worsening of an old symptom over at least 24 hours that could be attributed to MS. Sustained disability progression was defined as an increase in EDSS score of at least one point at year one compared to baseline.

**Statistical analysis**

SPSS 16.0 for Windows was used for statistical analysis of clinical and demographic data. Spearman correlation coefficient was used to assess the correlation between antibody and serum natalizumab concentrations. Logistic regression analyses were performed to investigate the relationship between natalizumab concentrations, antibody titers and
clinical (relapses dichotomized) and radiological (Gd+ lesions dichotomized) parameters. To analyse these responses, we used the serum natalizumab concentration and natalizumab antibody titers at week 24, or week 12 in some cases if this sample was missing. Both serum and antibody concentrations, were analysed as categorical variables in the regression analyses. Results of the regression analysis are presented as odds ratios (OR) with 95% confidence intervals (CI). All reported p values are based on two-tailed statistic tests, with a significance level set at <0.05.

Results

Patient characteristics

From March 2007 to March 2009, 73 patients were included and were followed up until March 2010. The median number of infusions with natalizumab was 13 (range 3-39 infusions). The mean disease duration before starting natalizumab was 112 months (SD 72) (Table 1).

Three patients were lost to follow-up in the first year as they received their subsequent natalizumab infusions in other hospitals after receiving the first infusions at the MS Centre of the VU University Medical Centre.

Four patients stopped natalizumab therapy prematurely during the first year of treatment. Three patients stopped due to side effects, two because of an allergic reaction, and one patient stopped because of clinical and radiological disease activity. All patients who stopped therapy early had clinical and radiological follow-up.

Natalizumab antibody development during natalizumab therapy

In our cohort, 31 out of the 73 tested patients (42%) had only antibody negative samples during follow-up and 42 patients (58%) tested antibody positive at least at one single time-point during the study. Of these 42 positive patients, 24 (33% of all patients) had low antibody titers at all time points, whereas 18 (25% of all patients) had high antibody titers (range 110–260,000 AU/ml) at least at one time point during the study. All patients who developed anti-natalizumab antibodies developed them before week 24. From all patients for whom serum was available at one year after start of the natalizumab therapy, the large majority was anti-natalizumab antibody negative (95.4%) (Figure 1). Of the two patients with an allergic reaction in the first year, one patient had no detectable serum natalizumab concentration with a very high antibody
concentration at week 12 (45,000 AU/ml). The other patient had no antibodies at week 4 and had a remarkably high serum natalizumab concentration (150,000 µg/ml). The patient who stopped because of clinical and radiological activity had very low natalizumab concentration (<0.001-0.10 µg/ml) with very high anti-natalizumab antibody titers (range 80,000-260,000 AU/ml). Another patient with low serum natalizumab concentrations and high anti-natalizumab antibodies at multiple time points stopped after 27 infusions because of clinical and radiological activity. Interestingly, after cessation of natalizumab, an increase in antibody concentrations was seen, probably due to a decrease in immune complex formation (Figure 2).

**Serum natalizumab concentration during natalizumab therapy**

At week 12, median serum natalizumab concentration was 22 µg/ml and varied from undetectable to 210 µg/ml. At week 24, median serum natalizumab concentration was 31 µg/l and varied from undetectable to 220 µg/ml. Serum natalizumab concentration was reduced in patients with natalizumab antibodies and even more in patients with high antibody titers. The presence of antibodies was inversely correlated with the serum natalizumab concentration (correlation coefficient r = -0.765, p<0.001) (Figure 3).

**Effect of serum natalizumab concentrations and anti-natalizumab antibodies on MRI outcomes**

At baseline, 47 patients (65.3%) had one or more Gd+ lesions. However, at year one only six patients (8.2%) had Gd+ lesions. Three of them had very low natalizumab concentrations in combination with positive anti-natalizumab antibody titers (Figure 4 and 5), and two of these patients also suffered a clinical relapse. The other three patients had normal serum natalizumab concentrations and no antibodies. Remarkably, two out of these three patients showed an extensive number of Gd+ lesions on their MRI scan at baseline (26 and 35 Gd+ lesions, respectively). Logistic regression showed a significant positive effect for patients with natalizumab concentrations <1.0 µg/ml on the presence of Gd+ lesions (OR 14.5, 95% CI 2.2-96.4, p=0.006) (Figure 4). High antibody titers have a quite similar effect on the presence of Gd+ lesions compared to no antibodies (OR 10.5, 95% CI 1.6-70.3, p=0.02) (Figure 5). No difference in the presence of Gd+ lesions was found between patients with low antibodies compared to patients with no antibodies. Neither the serum natalizumab concentration nor the presence of antibodies was
significantly correlated to new or enlarged T2 lesions. In summary, both low natalizumab concentrations and high antibody titers at week 24 are associated with the occurrence of Gd+ lesions during natalizumab treatment at year one.

**Effect of serum natalizumab concentration and anti-natalizumab antibodies on relapses and EDSS**

Regarding the clinical impact of serum natalizumab concentration, patients with a serum natalizumab concentration below 1.0 µg/ml showed 9.0 times higher odds of having a relapse (OR 9.0, 95% CI 1.7-47.9, p=0.01) compared to patients with a serum natalizumab concentration ≥1.0 µg/ml (Figure 4). The odds of having a relapse were also significantly higher in patients with high antibody concentration compared to the patients with no antibodies (OR 10.9, 95% CI 1.9-63.6, p=0.008) (Figure 5). Also, patients with a high antibody titer and simultaneously low serum natalizumab concentration had a 13.2 times higher odds on a relapse compared to patients with no antibodies and a serum natalizumab concentration ≥1.0 µg/ml (OR 13.2, 95% CI 2.1-84.5, p=0.006). Of the four patients that had serum natalizumab concentrations <0.001 µg/ml at any moment during the study, all had suffered from one or more relapses, and two of them had Gd+ lesions as well. Also, one of these patients had an allergic reaction. No correlation between antibodies or natalizumab levels was found with disability progression.

In summary, both low natalizumab concentrations and high antibody titers at week 24 are associated with the occurrence of relapses during natalizumab treatment at year one.

**Discussion**

The combined analysis of natalizumab serum concentration and antibodies suggests that both low natalizumab concentration and persisting antibody positivity at week 24 are associated with a lack of efficacy of natalizumab. In addition, a clear inverse correlation between natalizumab serum concentration and antibodies was found. It is well described that levels of antibody formation against different therapeutic proteins vary between products and laboratory methods used and have been reported previously for natalizumab to range between 4.5-14.1%. Here, we found a substantially higher percentage of at least once positive patients with anti-natalizumab antibodies (58%) than previously reported. This difference in transiently positive patients is most likely...
due to differences in methods used. The RIA method that we used seems more suited than the ELISA to detect anti-natalizumab antibodies in serum when free natalizumab is circulating in the serum as well, as was previously observed in a study that compared the RIA and ELISA methods to detect anti-adalimumab antibodies. Our data did not reveal the clinical relevance of transiently positive antibody measurements. Presumably, the low antibody titers, possibly in combination with a relatively low affinity, are insufficient to significantly affect natalizumab concentrations.

Antibodies against natalizumab can form natalizumab-anti-natalizumab immune complexes. One possible hypothesis is that when these immune complexes are formed, antibodies are not detectable in the serum anymore. This is clearly illustrated in the patient who stopped natalizumab treatment and showed a subsequent increase in antibody concentration afterwards (Figure 2). The formation of these immune complexes also leads to an increase of clearance of natalizumab and thus results in a low serum natalizumab concentration. This concept is supported by observations in patients with rheumatoid arthritis receiving technetium-99 labelled infliximab. The decrease in functional serum concentrations of the protein is most likely the cause of a loss of efficacy of natalizumab.

So far, immunogenicity studies in natalizumab patients have focused mainly on neutralizing antibodies. We here show that low serum natalizumab concentrations predict lack of clinical and radiological efficacy with at least the same precision as antibodies. The measurement of serum natalizumab concentrations is much easier to standardize compared to antibody measurements, in addition to providing a direct measure of active drug in circulation. Besides that, we saw that the three patients with a serum natalizumab concentration less than 0.01 µg/ml, all were clinically active. In addition to measuring the amount of anti-natalizumab antibodies, the circulating serum natalizumab concentration may provide further guidance during natalizumab treatment. For example, when natalizumab concentrations are increasing, it may be considered appropriate to continue natalizumab infusions even though there are high antibody concentrations and it may still be possible to reach a functional natalizumab concentration with clinical and radiological stability. In the one patient with high antibody concentrations who stopped natalizumab treatment after two years, we eventually noticed that just before discontinuation of therapy, a functional natalizumab concentration might have been reached. If patients with a <1.0 µg/ml natalizumab
concentration in their serum (who have not experienced severe hypersensitivity reactions to the drug) are given higher doses of natalizumab or more frequent infusions, functional levels may potentially be reached that would not have been reached with the standard dose of 300 mg given intravenously every four weeks. Future studies are needed to confirm clinical applicability.

In this study, all patients developed their antibodies by week 24. Almost all patients in the AFFIRM and SENTINEL trials who developed anti-natalizumab antibodies, 55 and 70 patients, respectively (97 and 100%, respectively), exhibited detectable antibodies by week 24.11 All nine patients in the study of Oliver et al., and all 30 patients in the study of Jensen et al., developed their antibodies before 16 weeks and three months, respectively.12,14 In all these studies almost all of the patients who developed antibodies, it was seen that they developed them early after the initiation of their treatment before week 24. This suggests that measurement of antibodies after this time in patients who are antibody negative at week 12 and 24 is of little importance. One possible exception might be patients with adverse drug reactions or late hypersensitivity reactions.

A relatively small fraction of anti-natalizumab patients appears to be long-term positive. The majority of these patients had high antibody titers. Although only three out of 65 patients (4.6%) had anti-natalizumab antibodies after one-year follow-up, it must be noted that of the seven patients who discontinued natalizumab treatment or were lost to follow-up, four patients had high antibody titers at the last moment of testing.

It is currently unknown why most patients develop only transient antibodies and why in some patients (mainly those with high antibody titers) antibodies persist. Such results suggest that for many patients the absence of levels of antibodies large enough to cause loss of efficacy may be the result of the development of tolerance rather than immunological neglect. This should be further explored.

The serum natalizumab concentration at week 24 correlates well with the presence of anti-natalizumab antibodies. Very low or undetectable serum natalizumab concentrations are associated with a high antibody concentration and both are associated with more relapses and more Gd+ lesions on MRI at year one. It must be noted, however, that the number of patients investigated was relatively small as was the number of relapses and Gd+ lesions and that these results have to be confirmed in a larger group of patients.
Lastly, the large variation in natalizumab levels that was measured in patients who responded well suggests that a dose reduction might be feasible for some patients, and also that dosage regimens could be based on therapeutic drug monitoring. This, however, requires further investigation.

Altogether we here confirm the correlation between high anti-natalizumab antibodies and a lack of efficacy of natalizumab. In addition, our data suggest possible clinical relevance of measuring drug levels, especially in those patients who do not show an optimum response to the drug during the first year of treatment. So, measuring serum natalizumab concentration might in the future lead to more enhanced precision in using natalizumab in individual patients.
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(19) van der Laken CJ, Voskuyl AE, Roos JC, et al. Imaging and serum analysis of immune complex formation of radiolabelled infliximab and anti-infliximab in
<table>
<thead>
<tr>
<th><strong>Table 1: Demographic and clinical characteristics</strong></th>
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<tr>
<td><strong>N</strong></td>
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<tr>
<td><strong>Sex (% female)</strong></td>
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<tr>
<td><strong>Age at start natalizumab (years)</strong></td>
</tr>
<tr>
<td><strong>Disease duration at start natalizumab (months)</strong></td>
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<tr>
<td><strong>Previous DMT last two years (n,%)</strong></td>
</tr>
<tr>
<td>Interferon-bêta (IFN-β)</td>
</tr>
<tr>
<td>Glatiramer-acetate (GA)</td>
</tr>
<tr>
<td>IFN-β + GA</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td><strong>Annualized relapse rate (ARR)</strong></td>
</tr>
<tr>
<td>Within 12 months before natalizumab</td>
</tr>
<tr>
<td>Within 24 months before natalizumab</td>
</tr>
<tr>
<td><strong>EDSS at start natalizumab (median, IQR)a</strong></td>
</tr>
<tr>
<td>0-3.5 (n, %)</td>
</tr>
<tr>
<td>≥ 4.0 (n, %)</td>
</tr>
<tr>
<td><strong>Magnetic Resonance Imaging at start natalizumab (n, %)</strong></td>
</tr>
<tr>
<td>&gt;9 T2 hyper intense lesions at baseline (n = 73)</td>
</tr>
<tr>
<td>Gadolinium enhancing lesions at baseline (n = 71)b</td>
</tr>
<tr>
<td><strong>Follow up duration during natalizumab (n, %)</strong></td>
</tr>
<tr>
<td>&lt; 1 year</td>
</tr>
<tr>
<td>1 year</td>
</tr>
<tr>
<td>2 years</td>
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<td>3 years</td>
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Data presented as mean ± SD unless otherwise indicated. 

a EDSS (Expanded Disability Status Scale) at start natalizumab: 3/73 missing. IQR = Interquartile Range 
b Gadolinium at start natalizumab: 2/73 missing
Figure 1. Antibody status during the first year. Available antibody status of all 73 patients at 12, 24 and 52 weeks (3, 6 and 13 infusions, respectively). It must be noted that of the seven patients who discontinued natalizumab treatment or were lost to follow-up, four patients had high antibody titers at the last moment of testing. From one patient with a high serum antibody titer until week 36, serum was lacking at week 52. The 13 samples missing at week 12 were from different patients than the five samples missing at week 24.

Ab: antibody; AU: arbitrary units
Figure 2. Anti-natalizumab antibody and serum natalizumab concentrations from one patient with high antibody titers during two years of natalizumab infusions and after discontinuation of the natalizumab infusions. The antibody titer was decreasing over the time and serum natalizumab concentration was slowly increasing. After cessation of natalizumab, the antibody concentration was increasing probably due to a decrease in immune complex formation.

AU: arbitrary units
Figure 3. Scatter plot showing the correlation between anti-natalizumab antibodies and serum natalizumab concentration at week 24 (Spearman’s Rho -0.765, p<0.001) in all patients with anti-natalizumab antibodies.

AU: arbitrary units
**Figure 4.** Low serum natalizumab concentrations have a 14.5 times higher odds ratio (OR) of 14.5 (95% confidence level (CI) 2.2-96.4, \(p=0.006\)) to develop gadolinium positive (Gd+) lesions and a nine times higher odds (OR 9.0, 95% CI 1.7-47.9, \(p=0.01\)) to have a relapse compared to normal serum natalizumab concentrations.
Figure 5. Patients with a high antibody titer have a 10.5 times higher odds ratio (OR) of 10.5 (95% confidence level (CI) 1.6-70.3, $p=0.02$) to develop gadolinium positive (Gd+) lesions and a 10.9 times higher odds (OR 10.9, 95% CI 1.9-63.6, $p=0.008$) to have a relapse compared to patients with no antibodies.
Chapter 2.2

Natalizumab drug holiday in MS: poorly tolerated

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**Abstract:** It has been suggested that natalizumab-associated progressive multifocal leukoencephalopathy may be prevented by structured interruptions of treatment. Evidence supporting such a drug holiday is not yet available. Here we present initial observations in 10 multiple sclerosis patients who were stringently monitored up to 6 months after discontinuation of the infusions. Cumulatively, a combination of clinical relapse and new and/or enhancing lesions on magnetic resonance imaging had occurred in 7 out of 10 patients. Although numbers are small, our data suggest that in patients who were switched to natalizumab because of disease activity despite first-line treatment, a natalizumab drug holiday without reinstatement of alternative disease-modifying therapy is poorly tolerated.
Natalizumab is a monoclonal antibody with specificity for alpha-4 integrin that inhibits the entry of mononuclear cells to sites of inflammation in the central nervous system. It has been approved worldwide for treatment of relapsing-remitting multiple sclerosis (MS) based on its efficacy in phase 3 trials.\(^1,2\) Use of natalizumab is associated with a rare risk of development of progressive multifocal leukoencephalopathy (PML), a potentially lethal demyelinating disease of the central nervous system caused by lytic infection of oligodendrocytes by the John Cunningham virus (JCV). As of January 2010, natalizumab has been prescribed to >50,000 individuals with MS, and the number of confirmed PML cases has now risen to >30 worldwide.\(^3\) Although details on many of these cases have not been published, the US Food and Drug Agency concluded in September 2009 that the risk for PML appears to increase with the number of natalizumab infusions received, the current rate ranging from 0.4 to 1.3 per 1,000 patients who have received at least 24 infusions.\(^4\) In January 2010 the European Medicines Agency recommended additional measures to better manage risk of PML with natalizumab.\(^3\) Already in 2007, accompanying the worldwide introduction of natalizumab, Kappos and colleagues provided recommendations for patient selection and monitoring, including diagnostic algorithms to apply in natalizumab-treated patients with clinical or magnetic resonance imaging (MRI) findings suggestive of PML.\(^5\) However, clinical vigilance is the most important indicator of suspected PML, because laboratory markers that predict the likelihood for PML are not yet available. Findings on whether JCV DNA is increased in plasma and peripheral blood mononuclear cells after natalizumab therapy are inconsistent, and there is no firm evidence that JCV DNA can predict which patients ultimately develop PML.\(^6-8\)

In addition to focusing on early recognition of PML, some experts have questioned whether PML can be prevented by using natalizumab in treatment cycles with limited duration or whether structured interruptions of treatment should be instituted.\(^9,10\) Evidence supporting such a drug holiday is not yet available, neither on its most appropriate timing nor on its most appropriate duration. It can, however, be assumed that such duration should be at least 3 months, because it has been shown that it takes about this amount of time for natalizumab serum concentrations to drop below 1 μg/ml, a level below which desaturation of alpha4-integrin is being observed.\(^11\)

Here we report initial observations in 10 patients with relapsing MS who after 12 months of treatment had a favorable response to the drug, both clinically and on MRI,
and thereafter decided to discontinue treatment for a variety of reasons. Clinical and MRI assessments were prospectively planned to be performed 3 and 6 months after discontinuation of monthly infusions.

**Patients and methods**

Since 2006, about 100 patients have started treatment with intravenous natalizumab (Tysabri®, Biogen Idec, Cambridge, MA, USA) 300mg every 4 weeks at the MS Center of the VU Academic Medical Center in Amsterdam, the Netherlands. These patients all had relapsing MS that was clinically active (2 relapses, or 1 relapse and new lesions on brain MRI during the past year) despite treatment with first-line disease-modifying agents (interferon beta or glatiramer acetate). Patients had monthly visits for relapse assessment, while EDSS and brain MRI were performed at baseline and every year thereafter.

From those patients who had a favorable response after 1 year of treatment (no relapses, not more than one new T2 lesion on 1 year MRI compared to baseline, no gadolinium-enhancing lesions at 1 year MRI), several have now discontinued treatment, for a variety of reasons. Patients who discontinued natalizumab agreed to have clinical visits and gadolinium-enhanced brain MRI at treatment discontinuation and thereafter at 3-months intervals (MRI was only performed if patients were not pregnant). Here we report on the first 10 patients who were monitored according to the aforementioned algorithm after their discontinuation of treatment.

Patient characteristics are given in the Table. All patients had been pretreated with interferon beta. Three had also been on glatiramer acetate. Their mean age at start of treatment with natalizumab was 39 years. They discontinued natalizumab after a mean treatment duration of 23 months (from 12 to 40 months). Reasons for discontinuation were desire to become pregnant in 2 patients, subjective disease worsening in 1 (in absence of relapse, or objective EDSS worsening, or new lesional activity on MRI), and fear for future risk of PML in 7.

**Results**

At discontinuation of treatment all patients were clinically stable; none of them revealed new lesions on their MRI in comparison to the last MRI on treatment.
At 3 months, 2 patients had clinical activity, and 3 had new and/or enhancing lesions on brain MRI (see Table). The patient who discontinued treatment because of subjective worsening had a severe clinical relapse with EDSS worsening of >1 full point 12 weeks after discontinuation; his MRI showed many new lesions that were enhanced after administration of gadolinium contrast (Fig). The other patient had a clinical relapse 2 months after her last natalizumab infusion; her MRI showed a big gadolinium-enhanced lesion. These patients both opted to restart treatment with natalizumab. At 6 months, of the remaining 8 patients, 5 had had a clinical relapse, and all of these patients also had new and/or enhanced lesions on brain MRI. Of the 3 patients who were clinically well, 2 had become pregnant (no MRI performed at month 6 because of pregnancy).

Cumulatively, at the end of 6 months, a combination of clinical relapse and new and/or enhanced lesions on MRI had occurred in 7 of 10 patients (see Table). The mean interval between treatment discontinuation and occurrence of relapse in these patients was 17 weeks (range 8 to 22 weeks). All 7 active patients opted to restart with natalizumab.

**Discussion**

In 10 consecutive patients who responded well to natalizumab infusions, but decided to discontinue for a variety of reasons (mainly fear for PML risk and desire to become pregnant), we observed clinical relapses and accompanying active lesions in 7 at 6 months after discontinuation of natalizumab. Only 3 patients (2 of whom had become pregnant) were still clinically well at 6 months, although 1 of the patients who became pregnant had had new T2 lesions on MRI at month 3. Strikingly, and well in line with pharmacokinetic data, disease activity started to occur about 3 months after discontinuation of natalizumab.

In our view, although the patient number is quite limited, these data strongly suggest that in patients who were switched to natalizumab because of disease activity on first-line immune modulatory therapy and responded well, discontinuation of natalizumab is poorly tolerated. None of the patients received other disease-modifying drugs after discontinuation of natalizumab, because they all had failed on first-line immunomodulatory treatment and starting immunosuppressive drugs was assumed to keep the risk of PML elevated. In this small observational study, a drug holiday of 6 months leads to undesired disease activity in the large majority of patients; by that time
7 of 10 patients had received treatment with intravenous steroids because of a relapse and these patients all opted for immediate restart with natalizumab. Although the extent of clinical and radiological disease activity after discontinuation of natalizumab is striking, the limited sample size and the amount of disease activity before natalizumab initiation do not allow interpretation of these data as evidence for an overshoot of disease activity compared to the prenatalizumab treatment period. None of the patients showed clinical or radiological signs and symptoms that were suspect for either active PML or an immune reconstitution inflammatory syndrome. For these patients, who probably represent a subgroup with active disease because they all had documented clinical disease activity while being on first line therapy, we do not recommend discontinuation of natalizumab as a drug holiday without reinstatement of alternate disease-modifying therapy.
References


3. Questions and answers on the review of Tysabri (natalizumab).

4. Information on Natalizumab (marketed as Tysabri).


### Table: Clinical and Radiological Characteristics of 10 Relapsing-Remitting MS Patients Who Discontinued Natalizumab

<table>
<thead>
<tr>
<th>Patients(^a)</th>
<th>Months(^b)</th>
<th>Reasons discont.</th>
<th>Relapses year prenatalizumab</th>
<th>Baseline MRI prenatalizumab</th>
<th>Clinical Month 3 after discont.</th>
<th>MRI Month 3 after discont.</th>
<th>Clinical Month 6 after discont.</th>
<th>MRI Month 6 after discont.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F, 53</td>
<td>27</td>
<td>Fear of PML</td>
<td>2 exacerbations</td>
<td>No active lesions</td>
<td>Exacerbation</td>
<td>1 large enhancing lesion + 1 new T2</td>
<td>Stable after IVMP and restart natalizumab</td>
<td>No active lesions</td>
</tr>
<tr>
<td>F, 29</td>
<td>28</td>
<td>Fear of PML</td>
<td>1 exacerbation</td>
<td>3 new T2, no enhancing lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Exacerbation treated with IVMP</td>
<td>25 enhancing lesions</td>
</tr>
<tr>
<td>F, 26</td>
<td>22</td>
<td>Fear of PML</td>
<td>4 exacerbations</td>
<td>26 enhancing lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Exacerbation treated with IVMP</td>
<td>19 enhancing lesions</td>
</tr>
<tr>
<td>F, 34</td>
<td>13</td>
<td>Desire to become pregnant</td>
<td>3 exacerbations</td>
<td>2 new T2, 1 enhancing lesion</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Pregnant, stable</td>
<td>Not done</td>
</tr>
<tr>
<td>F, 41</td>
<td>22</td>
<td>Fear of PML</td>
<td>1 exacerbation</td>
<td>No active lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Exacerbation treated with IVMP</td>
<td>3 enhancing lesions</td>
</tr>
<tr>
<td>M, 48</td>
<td>40</td>
<td>Fear of PML</td>
<td>0 exacerbations</td>
<td>2 new T2 lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Stable</td>
<td>No active lesions</td>
</tr>
<tr>
<td>F, 49</td>
<td>23</td>
<td>Fear of PML</td>
<td>2 exacerbations</td>
<td>6 new T2, 2 enhancing lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Exacerbation treated with IVMP</td>
<td>4 enhancing lesions</td>
</tr>
<tr>
<td>M, 51</td>
<td>21</td>
<td>Subjective worsening</td>
<td>1 exacerbations</td>
<td>5 new T2, 3 enhancing lesions</td>
<td>Exacerbation</td>
<td>&gt; 50 enhancing lesions</td>
<td>Stable after IVMP and restart natalizumab</td>
<td>1 enhancing lesion, no new T2</td>
</tr>
<tr>
<td>F, 26</td>
<td>12</td>
<td>Desire to become pregnant</td>
<td>2 exacerbations</td>
<td>3 enhancing lesions</td>
<td>Stable</td>
<td>2 new T2 lesions, no enhancing lesions</td>
<td>Pregnant, stable</td>
<td>Not done</td>
</tr>
<tr>
<td>F, 41</td>
<td>24</td>
<td>Fear of PML</td>
<td>2 exacerbations</td>
<td>4 new T2, 3 enhancing lesions</td>
<td>Stable</td>
<td>No active lesions</td>
<td>Exacerbation treated with IVMP</td>
<td>4 enhancing lesions</td>
</tr>
</tbody>
</table>

Baseline MRI prenatalizumab: scan performed during first-line disease modifying-treatment (interferon-beta or glatiramer acetate) within 3 months before start natalizumab. New T2 lesions compared to baseline scan before initiation first line treatment. Active lesions: new T2 and/or gadolinium-enhanced lesions.

\(^a\)Gender, age in years at the time of initiation of natalizumab therapy
bNumber of natalizumab infusions
MS = multiple sclerosis; MRI = magnetic resonance imaging; F = female; PML = progressive multifocal leukoencephalopathy; IVMP = intravenous methylprednisolon 1000 mg for 3 days; M = male
Figure: T1-weighted postgadolinium contrast and T2-weighted images just before (a, c) and 12 weeks after (b, d) discontinuation of natalizumab in a patient (male, aged 51 years) who discontinued treatment after 21 infusions (completely stable Expanded Disability Status Scale [EDSS] and magnetic resonance imaging [MRI] without new lesions during natalizumab treatment). He had a severe clinical relapse with EDSS worsening of >1 full point. His MRI showed > 50 new enhanced lesions (b) and new T2 lesions (d) 12 weeks after discontinuation of natalizumab, whereas no enhancement (a) was observed just before discontinuation.
Chapter 2.3

Natalizumab remains detectable in MS patients long after treatment is stopped

Theo Rispens, PhD1; Anke Vennegoor, MD2; Gert Jan Wolbink, MD, PhD1; Chris H. Polman, MD, PhD2; Joep Killestein, MD, PhD2

Multiple Sclerosis Journal 2012;18(6):899-901
Abstract

Natalizumab is frequently used as a treatment of multiple sclerosis (MS). The occurrence of progressive multifocal leukoencephalopathy (PML) in natalizumab-treated patients indicates that its prominent beneficial effects need to be balanced against the risks. Also, cessation of the drug seems to be associated with recurrence of disease activity. Both the moment of rebound disease activity and the outcome of PML are related to clearance of the drug. Specific features of this IgG4 antibody (i.e. half-antibody exchange) may result in underestimated drug levels. Here, we demonstrate natalizumab levels in 10 patients with relapsing MS, using a recently developed sensitive assay. Remarkably, natalizumab was detectable up to 200 days after cessation of therapy.
Introduction

Natalizumab (Tysabri, Biogen Idec, Inc., and Elan Pharmaceuticals, Inc.), a recombinant antibody directed against alpha4-integrin, is frequently used as a treatment of multiple sclerosis (MS). Phase III trial data has shown that natalizumab dramatically reduces the frequency of relapses and MRI lesion formation in patients with relapsing MS.¹ In these studies, benefits were realized rapidly and persisted throughout the treatment period. However, the occurrence of natalizumab-associated progressive multifocal leukoencephalopathy (PML) in natalizumab-treated patients indicates that these prominent beneficial effects need to be balanced against the risk of a potentially life-threatening adverse event.² ³ As of February 2011, 95 cases of natalizumab-associated PML have been reported in MS (www.biogen.com). Recent studies have suggested another hazard of exposure to natalizumab: cessation of the drug seems to be associated with recurrence of clinical and radiographic disease activity. Some of these exacerbations were clinically severe, with a high number of contrast-enhanced lesions, suggesting a rebound of disease activity. This rebound occurs somewhere between 2 and 6 months after cessation of therapy.⁴ ⁵ Although it is unclear what determines the point in time of the rebound after cessation of therapy, obviously, this may largely depend on the swiftness of clearance of natalizumab from the body. Both the moment of rebound disease activity and the outcome of PML are related to natalizumab levels and clearance of the drug. For proper management of PML, it is important to have insight in natalizumab levels after treatment interruption, to determine whether or not plasma exchange should be started and continued. However, clinical data on clearance of natalizumab are scarce. Until now, clearance of natalizumab has been monitored only for relatively short periods of time. For instance, extrapolating natalizumab levels monitored for 28 days, Khatri et al predict that it would take almost 100 days before median levels drop below 1 mg/l, a level below which partial desaturation of alpha4-integrin was observed.⁶ Natalizumab levels after administration of a single dose were monitored for up to 8 weeks,⁷ ⁸ but this does not represent patients receiving repeated doses of natalizumab.

Here, we present data of natalizumab levels for up to 260 days after stopping treatment in 10 patients with relapsing MS who, after at least 12 months decided to discontinue treatment for a variety of reasons.
**Patients and methods**

Since 2006 about 115 patients have started treatment with intravenous natalizumab 300 mg every 4 weeks, at the MS Centre of the VU Academic Medical Centre in Amsterdam, the Netherlands. Several have now discontinued treatment, for a variety of reasons. Here we further report on the first 10 patients who were monitored according to an aforementioned algorithm (frequent MRI and clinical assessments) after their discontinuation of treatment. Their mean age at start of treatment with natalizumab was 39 years. They discontinued natalizumab after a mean treatment duration of 23 months (range 12–40 months). Serum samples were drawn under informed consent. Blood samples were obtained just before the last dose of natalizumab was received and at different time points thereafter. These patients have been described in more detail elsewhere.

We recently developed a sensitive assay to determine serum levels of natalizumab that takes into account several specific features of natalizumab. Natalizumab is an IgG4 antibody, which can undergo Fab arm exchange (half-antibody exchange), resulting in antibodies containing a single natalizumab-derived antigen-binding site. A bridging ELISA using natalizumab-specific antibodies is therefore not suited to quantify drug levels (but could be used to detect only bivalent natalizumab). However, alternative assay formats require careful assay design to avoid high background signals due to non-specific binding of serum IgG4 to coated (anti-idiotypic) antibodies. We solved this problem by using F(ab’)2 fragments of anti-idiotypic rabbit antibodies as capture reagent. The resulting assay is able to detect natalizumab down to 10 ng/ml in serum, i.e. substantially more sensitive compared to other published assays. Precision of the assay is 13% for bivalent natalizumab and 17% for ‘monovalent’ natalizumab, respectively, and accuracy is 105% and 112%. To prepare ‘monovalent’ natalizumab, a tenfold excess of an irrelevant IgG4 antibody was incubated with natalizumab at 37°C in the presence of 0.5 mM GSH to induce Fab arm exchange.

**Results**

Natalizumab levels were evaluated and the results are presented in Figure 1. Median concentration dropped from 25 (range 18.4–104) mg/l during treatment to 0.28 (range 0.1–1) mg/l 3 months after the last dose was administered. In some patients,
natalizumab was detectable (>0.01 mg/l) even after 200 days. None of these patients had detectable anti-drug antibody levels (manuscript in preparation).

Discussion

Using a recently developed sensitive assay we measured natalizumab levels in 10 patients with relapsing MS who had discontinued natalizumab therapy. Natalizumab was detectable up to 200 days after cessation of therapy. Thus, it can take a substantial amount of time before natalizumab levels drop to values that no longer block alpha4-integrin. Also, considerable variation in natalizumab levels was found between individuals, suggesting the need for monitoring by measurement of actual drug levels in specific situations. Persisting natalizumab levels after cessation of therapy might have clinical implications; for example, indolent PML has been described followed by a very late but severe immune reconstitution inflammatory syndrome (IRIS). Interestingly, we measured blood levels of natalizumab at different time points during the course of PML and IRIS in this MS patient. The diagnosis was confirmed late, more than two months after stopping natalizumab, and therefore she was not treated with plasma exchange or immunoabsorption. Also, the patient had shown some initial spontaneous improvement after cessation of therapy. Nevertheless, she developed severe IRIS. We measured serum levels of 1.1 mg/l 10 weeks after stopping natalizumab during the phase of clinical improvement, 0.6 mg/l during the phase in which IRIS became clearly visible on MRI 2 weeks later and 0.1 mg/l 2 weeks thereafter, when the patient’s clinical condition had worsened due to IRIS and IV methylprednisolone was started after unsuccessful treatment with IV immunoglobulins.

Another suggestion of possible clinical relevance of measuring drug levels in our study was found in the fact that rebound disease activity occurred later in patients who showed the highest natalizumab levels. Unfortunately, the small group size and limited temporal resolution did not allow a more rigorous analysis. Accurate drug level measurement may provide guidance in when to start timely alternative treatment in patients switching from natalizumab. Identification of the best moment to start alternative treatment after cessation of therapy will be one of the main objectives of recently developed studies (e.g. the RESTORE study, clinicaltrials.gov NCT01071083).

We conclude that accurate measurement of natalizumab levels may contribute to a better understanding of the underlying mechanisms and the management of
natalizumab-associated PML and the occurrence of rebound disease activity after cessation of the drug. Further research is needed, including functional assays, e.g. lymphocyte migration correlated to natalizumab serum levels as measured by a sensitive assay, and longitudinal serum level monitoring in both PML/IRIS cases, and larger cohorts of patients need to be sampled after cessation of natalizumab.

Acknowledgements

The authors would like to thank Lucien Aarden for critical reading of the manuscript.
References


Figure I. Natalizumab levels after abrogation of treatment.
Indicated on the x-axis is the number of days after receiving the last dose of natalizumab. For several patients, no serum sample was available for t = 0 and drug levels for t = 0 were instead estimated by using a serum sample taken at an earlier time point during treatment. Dashed line indicates cut-off.
Chapter 3

Serological biomarkers and their relevance in natalizumab-associated PML in MS patients
Chapter 3.1

High cumulative JC virus seroconversion rate during long-term use of natalizumab

A. Vennegoor¹, J.A. van Rossum¹*, C. Leurs¹*, M.P. Wattjes², T. Rispens³, J.L.A.N. Murk⁴, B.M.J. Uitdehaag¹, J. Killestein¹

European Journal of Neurology 2016;23(6):1079-1085
Abstract

**Background and purpose:** John Cunningham virus (JCV) seropositivity is a risk factor for the development of natalizumab-associated progressive multifocal leukoencephalopathy (PML) in multiple sclerosis (MS) patients. When JCV seronegative patients seroconvert, their risk of developing PML increases. Limited longitudinal data exists about the seroconversion rate among natalizumab-treated relapsing remitting MS (RRMS) patients. Our objective was to evaluate the seroconversion rate in a large Dutch cohort of natalizumab-treated RRMS patients. Seroconversion was defined as at least two consecutive seropositive serum samples (or cessation of therapy after a single seropositive sample because of seropositivity) after initial seronegative testing.

**Methods and results:** In our study of 179 patients for whom longitudinal blood samples were available over a long period (median 4.2 year), anti-JCV antibody indexes were measured in 933 available samples. Eighty-six patients (48.0%) tested seronegative initially. Of these 86 seronegative patients, 23 patients (26.7%) seroconverted during follow-up. The annualized seroconversion rate was 7.1%. Seroconversion occurred between 9 and 90 months (median 43 months) of treatment. The rate of seroconversion was independent of follow-up duration. No significant increase was seen in the anti-JCV antibody index in the non-converting patients during the follow-up.

**Conclusion:** The annualized seroconversion rate of 7.1% in patients using natalizumab, cumulatively leading to more than 25% of seronegative patients becoming seropositive in 4 years, is of clinical relevance and should be taken into account in the risk assessment when considering the start of natalizumab therapy.
**Introduction**

Natalizumab is a humanized monoclonal antibody against α4-integrins. It binds to the α4 chain of α4β1 and α4β7 integrin and blocks the adhesion to the vascular endothelium (VCAM-1 and MAdCAM-1) [1]. By this mechanism, natalizumab prevents leukocytes migrating into the central nervous system and thereby suppresses the inflammatory reaction in patients with multiple sclerosis (MS). Natalizumab is a well tolerated and highly efficacious drug in relapsing remitting MS (RRMS) [2], but can be complicated by progressive multifocal leukoencephalopathy (PML). This is a rare, but potentially life-threatening opportunistic infection of the brain caused by reactivation and replication of the John Cunningham virus (JCV). JCV seropositivity is a relevant risk factor for the development of PML. In JCV seronegative MS patients, the risk of developing PML is very low [2–4]. However, patients who are initially tested seronegative can be infected with the JCV and seroconvert during natalizumab treatment resulting in an increased risk of developing PML. In addition, it has been reported that seronegative patients can be JCV DNA positive, suggesting that seroconversion can also be caused by reactivation of the virus already present in a patient [5–7]. Initially, Gorelik et al. [8] reported annually seroconversion rates of about 2% in RRMS patients treated for 48 weeks with natalizumab with a follow-up of 5 years. Higher conversion rates were reported in several other papers [9,10]. Plavina et al. [9] reported a total seroconversion rate of 13% in 18 months in seronegative patients. Outteryck et al. [10] found a seroconversion rate of 26.7% in their seronegative patients in 1 year. The European Medicines Agency decided in May 2015 to investigate if the reported rate of 13% has to result in reconsidering the recommendation of retesting every 6 months [11].

The aim of this study was to evaluate the conversion rate in a large Dutch cohort of anti-JCV antibody negative natalizumab-treated RRMS patients with a long follow-up duration for whom longitudinal blood samples were available using the STRATIFY-2 test in all samples.

**Methods**

**Patients and materials**

All MS patients were included who started natalizumab in the MS Centre of the VU University Medical Centre in Amsterdam and for whom at least two serum samples available of which at least one was after natalizumab initiation. In total 179 patients
Serum samples were collected every 3 months from February 2007 until January 2015 and immediately stored at -80°C. Demographic data, disease duration, Expanded Disability Status Scale (EDSS) at baseline and data about previous treatments were collected. From all patients until January 2014 a baseline sample (preferably before the start of natalizumab) and samples regularly during follow-up until January 2015 were obtained. Masked for clinical characteristics all samples were tested in one run in an independent laboratory (Unilabs, Copenhagen, Denmark). Since February 2012, serum samples from all patients have been analysed for JCV more regularly, nowadays every 6 months. The second-generation JCV antibody enzyme-linked immunosorbent assay (STRATIFY-2 test) was used to determine the anti-JCV antibody indices in serum(12). Quantitative results expressed as serum antibody index values were received. Serum sample dilution was experimentally demonstrated for indices ≤3.0(12). Seroconversion was defined as at least two consecutive seropositive serum samples (or in the case of cessation of therapy, after a single seropositive sample if the reason of cessation was becoming seropositive) after initial seronegative testing. The study was approved by the local institutional board (reference 2014.256). Written informed consent was obtained from all participants.

**Statistical analysis**

Continuous variables were expressed as mean and standard deviation if normally distributed or as median and interquartile range (IQR) if not normally distributed. Categorical variables were expressed as numbers and percentages. Differences in age, disease duration and EDSS were tested with the independent sample t-test or with the Mann-Whitney U test if not normally distributed. For gender and previous therapies, differences were tested with the Pearson Chi-squared or Fisher’s exact test. Differences in anti-JCV antibody indices over time between paired samples were tested using the Student’s paired t-test.

Univariable logistic regression was used to analyse the influence of potential predictive factors on seroconversion rate. Potential predictive factors were age at first JCV test, gender, disease duration at first JCV test and prior use of immunosuppressive treatment. If predictors had a $P$ value ≤ 0.2, they were analysed in a multivariable logistic regression model. Results were reported with their 95% confidence interval (CI) and a
statistical significance level of 0.05. The statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS, Inc., Chicago, IL, USA).

Results

Up to January 2014, 193 MS patients started natalizumab treatment in the MS Centre in Amsterdam, and in January 2015 179 patients met the inclusion criteria. Anti-JCV antibody indices were measured in 933 available samples from February 2007 until January 2015 (median follow-up duration serum samples 52.5 months, range 10.0-95.0 months, IQR 30.0-76.0 months). Of these 179 patients, 86 patients (48.0%) were initially tested seronegative and 93 (52.0%) were tested seropositive. Of the initial 86 seronegative patients, 23 patients (26.7%) converted from seronegative to seropositive in a median follow-up period of 4.2 years (range 49 days - 12.7 years). In 17 patients seropositivity was confirmed in a subsequent sample. The remaining six patients discontinued natalizumab treatment after this seropositive sample because of the seropositivity. Another patient discontinued natalizumab because of slow disease progression over time. No subsequent serum samples were available for confirmation.

The annualized seroconversion rate for all anti-JCV-antibody negative patients was 7.1%. Of the 23 patients who seroconverted, 11 patients were initially seronegative and the remaining 12 patients had an intermediate anti-JCV antibody index and were considered seronegative on the basis of the confirmatory assay. At the time of seroconversion, of these 23 patients, 14 patients (60.9%) had an anti-JCV antibody index >0.9: four patients (17.4%) had an anti-JCV antibody index >0.9 but <1.2, two patients (8.7%) had >1.2 but <1.5, and eight (34.8%) had >1.5 (Fig. 1). Eventually, looking at the highest anti-JCV antibody index in the patients, 16 patients (69.6%) had an index >0.9 of whom 11 patients (47.8% of the total converted patients) had an index >1.5.

Seroconversion occurred between 9–90 months (median 43 months, IQR 17-60 months). There was no evidence that seroconversion rate decreased or increased over time (Fig. 2).

After seroconversion, the number of follow-up samples varied from 0 to 5 (median 1, IQR 0- 2) with repeated sample timing varying from 0 till 38 months (median 6 months, IQR 0-17 months).

The indexes at the first anti-JCV antibody test were significant higher in the patients who converted from seronegative to seropositive compared to those patients who remained
seronegative [median 0.220 (IQR 0.160-0.300) and 0.170 (IQR 0.140-0.233), respectively, \( P=0.043 \)].

Student’s paired t-test showed no significant increase in anti-JCV antibody index over time in the seropositive group who remained seropositive (mean first test 1.96, SD 1.11 vs. mean last test 2.11, SD 1.23, \( P=0.29 \)) and in the seronegative group who remained seronegative (mean first test 0.19, SD 0.08 vs. mean last test 0.19, SD 0.12, \( P=0.47 \)) (Fig. 3). In the four patients who developed PML, the median anti-JCV antibody index before and during natalizumab therapy was 3.04 with a range of 2.04-3.59(13). In patients with a high anti-JCV antibody index (> 3.0), indices are technically not likely to increase further by reaching the saturation level of the assay(9).

At baseline, the mean age was 37.5 years and 34.9 years in the patients who were seropositive and seronegative, respectively. In the seropositive patient group 63 patients (67.7%) were female and in the seronegative group 61 patients (70.9%). Twenty-two patients (23.7%) in the seropositive group used glatiramer acetate before starting PML. In the seronegative group this were 36 patients (41.9%) (Table 1).

Table 1. Demographic and clinical characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>JCV positive</th>
<th>JCV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=179</td>
<td>n=93</td>
<td>n=86</td>
<td></td>
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<tr>
<td>Age at first JCV test (years), mean (SD)</td>
<td>36.3 (9.0)</td>
<td>37.5 (9.1)</td>
<td>34.9 (8.8)</td>
</tr>
<tr>
<td>Gender, n (% female)</td>
<td>124 (69.3)</td>
<td>63 (67.7)</td>
<td>61 (70.9)</td>
</tr>
<tr>
<td>Disease duration at first JCV test (months)A</td>
<td>89.0 (43.0-140.0)</td>
<td>94.0 (44.0-147.0)</td>
<td>83.5 (43.0-129.5)</td>
</tr>
<tr>
<td>Follow-up duration serum samples (months)A</td>
<td>52.5 (30.0-76.0)</td>
<td>55.0 (29.0-76.0)</td>
<td>51.0 (31.3-76.0)</td>
</tr>
<tr>
<td>EDSS at baseline NTZA</td>
<td>3.5 (2.8-5.5)</td>
<td>4.0 (3.0-5.5)</td>
<td>3.25 (2.5-5.5)</td>
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<td>IFN, n (%)</td>
<td>137 (76.5)</td>
<td>74 (79.6)</td>
<td>63 (73.3)</td>
</tr>
<tr>
<td>Glatirameracetate, n (%)</td>
<td>58 (32.4)</td>
<td>22 (23.7)</td>
<td>36 (41.9)</td>
</tr>
<tr>
<td>Treatment naive, n (%)</td>
<td>12 (6.7)</td>
<td>8 (8.6)</td>
<td>4 (4.7)</td>
</tr>
<tr>
<td>prior immunosuppressive agents, N (%)</td>
<td>9 (5.0)</td>
<td>3 (3.2)</td>
<td>6 (7.0)</td>
</tr>
</tbody>
</table>

EDSS, Expanded Disability Status Scale; IFN, interferon-β; JCV, John Cunningham Virus; NTZ, natalizumab.
Data represent median values and in parentheses interquartile range (IQR). Independent sample t-test, Mann-Whitney U test, Pearson Chi-squared test and Fisher’s exact test were performed if applicable.

Logistic regression showed no significant effect of age [odds ratio (OR) 0.977, 95% CI 0.92-1.03, P=0.43] and gender (OR -0.37, 95% CI 0.25-1.93, P=0.48) on seroconversion. Also there was no effect on seroconversion of disease duration (OR 1.00, 95% CI 0.99-1.01, P=0.49) and prior immunosuppressive medication (OR 1.41, 95% CI 0.24-8.24, P=0.71).

There were five patients initially seropositive who reverted to seronegative during follow-up. All these patients had a low anti-JCV antibody index at baseline (range index 0.269-0.553).

At the last moment of serum sampling (median follow-up since start of natalizumab 4.2 years), 68 patients (38.0%) tested seronegative and 111 patients (62.0%) seropositive.

Discussion

In our longitudinal study of the JCV serostatus amongst 179 natalizumab-treated MS patients an annualized seroconversion rate of 7.1% was observed, independent of the duration of follow-up. Compared to previous reports, our study has two major strengths: the follow-up is longer than in most other studies and all samples were tested with the same STRATIFY-2 test, where others compared results of different tests(10).

Gorelik et al. reported that 43% of the RRMS patients treated for 48 weeks with natalizumab for whom they had serial samples collected over 5 years in their study were stable seropositive, and 39% were stable seronegative. They report an annual conversion rate of approximately 2%(8). Plavina et al. reported a total seroconversion rate of 13% in 18 months in seronegative patients. They defined seroconversion as changing serostatus at least once during these 18 months. Seroconversion rates of 3-4% in 18 months were reported using indices of <0.9 and <1.5, respectively(9). Subsequently, higher conversion rates were reported in several other papers(10),(14),(15). Outteryck et al.(10) found a seroconversion rate of 26.67% in their seronegative patients in 1 year, using the first generation STRATIFY test for the initial test and the STRATIFY-2 test for the second test 12 months later. The use of a different methodology for follow-up samples is clearly limiting their conclusions, since it is known that the STRATIFY-2 test has a higher sensitivity than the first generation
STRATIFY test(12). Two other studies reported seroconversion during a relatively short follow-up: Trampe et al.(14) reported that 19 of 194 patients (9.8%) converted from seronegative to seropositive during an average observation time of 7.7 months and also Warnke et al.(15) reported a seroconversion rate of 10.3% with a median time point between sampling of 12 months. The annualized conversion rate of 7.1% we found in our cohort of initially seronegative patients is in the same range as reported by others, but with a much longer follow-up (median follow-up 4.2 years, IQR 3.6 years). Very recently, the European Medicines Agency decided to start an investigation based on a reported seroconversion rate of 13% in natalizumab-treated MS patients to reassess whether current recommendations of retesting every 6 months should be reconsidered(11). Our belief is that their assessment should take into account annualized conversion rates and the fact that seroconversion rate is not decreasing with longer usage of the drug.

Our observed annualized conversion rate is substantially higher than the estimated natural history based incidence rates of about 1-2%(16). Overall, in the general population the prevalence of anti-JCV antibodies is 57.1-58.3% and is inclined to increase with increasing age(17,18). In most countries, seropositivity was about 50-60%. The Netherlands, together with Austria and Portugal, have a higher anti-JCV seropositivity of about 66%-70%, but the reliability of these estimates may be reduced due to the fact that all these countries had relatively small sample sizes(17).

Still, annualized seroconversion rates of 7.1% would result in a much higher proportion of seropositive people, more than the expected increase with increasing age. This higher than expected conversion rate in natalizumab-treated MS patients cannot be explained by a more sensitive test as Outteryck at al.(10) suggested, as in our study only the STRATIFY-2 test was used. In the patients who seroconverted, the anti-JCV antibody index at the first moment of testing was higher than in the seronegative patients who stayed seronegative. It is not totally excluded that this is (partly) caused by patients who were already infected (i.e. JCV DNA positive) but tested seronegative initially, since JCV DNA status of our patients was not available. Recently, the results of some small studies suggested that patients can have viremia without anti-JCV antibodies(5–7) more often than initially described by Rudick et al.(19), who concluded that this was a rare condition. Less likely though, these patient can also exist in the seronegative group in which the patients stayed seronegative during follow-up. Larger studies in which JCV
DNA and anti-JCV antibodies will be measured during a long follow-up should give an answer to the additional value of measuring JCV DNA. It is hypothesized that this higher than expected seroconversion rate might be caused by higher vulnerability caused by the use of natalizumab. This can be supported by previous findings in studies in humans and animals suggesting that the interaction between α4β7 and MAdCAM-1 is important in mediating leukocyte homing to gut mucosa(20). The two largest trials (ENACT-2 and ENCORE) showed a significant effect of response to natalizumab in patients with Crohn’s disease(21,22). It has been suggested that in natalizumab-treated MS patients fewer T- and B-cells are seen in the intestinal mucosa as well. This may result in less immunity in the upper gastrointestinal tract and as a consequence a higher risk to get a primary infection with the JCV. In addition Kohlmann et al.(23) found an extremely high incidence of rising anti-VZV immunoglobulin G levels in natalizumab-treated MS patients in contrast to age- and gender-matched healthy blood donors and to HIV-infected patients.

The annualized seroconversion rate of 7.1% leads to more than 25% of seronegative patients becoming seropositive in 4 years, which has to be taken into account when discussing the start of natalizumab with patients. In conclusion, even though seroconversion rates in the general population were not tested, the annualized seroconversion rate and the increase in seropositivity observed in our study both suggest that JCV seroconversion is substantially higher in MS patients treated with natalizumab compared to what is expected in the general population. Seronegative patients treated with natalizumab are at increased risk for seroconversion and the majority (69.6%) of converted patients will eventually reach indices above 0.9. These results have implications for assessing the risks of developing PML in seronegative patients in whom a start with natalizumab is considered and the careful and continuous monitoring of JCV status on treatment.

Acknowledgements

The STRATIFY JCV™DxSelect™ test and shipping of the samples are financially supported by Biogen Idec.
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Figure 1 All anti-JCV antibody indices at baseline and at time of seroconversion of the 23 patients in whom seroconversion occurred. Of these 23 patients, 14 patients (60.9%) had an anti-JCV antibody index >0.9: four patients (17.4%) have an anti-JCV antibody index >0.9 but <1.2, two patients (8.7%) >1.2 but <1.5, and eight (34.8%) had >1.5.
Figure 2 Seroconversion over time. There is no increase or decrease noticeable over time in the amount of seroconversion. The median time until seroconversion is 43 months (IQR 17-60 months).
Figure 3 The anti-JCV antibody index in all 179 patients. (a) A significant increases ($P=0.013$) is seen over time. (b) This increase is particularly caused by the increase of anti-JCV antibody indices of the patients who convert from seronegative to seropositive.
Chapter 3.2

Longitudinal JCV serology in multiple sclerosis patients preceding natalizumab-associated progressive multifocal leukoencephalopathy

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Abstract

The presence of anti-John Cunningham Virus (JCV) antibodies is a risk factor for the development of progressive multifocal leukoencephalopathy (PML) in MS patients treated with natalizumab. It has been suggested that an increase in serum anti-JCV antibody index precedes the development of PML. We here describe extensive longitudinal serum anti-JCV antibody indexes of four MS patients who developed PML. Anti-JCV antibodies were measured using the STRATIFY JCV™DxSelect™ test. All four patients had rather stable high anti-JCV antibody indexes in all samples obtained before developing PML. Possibly caused by reaching the saturation level of the assay, no increase in anti-JCV antibody indexes was seen just before the diagnosis of PML. This study confirms that high serum anti-JCV antibody indexes precede natalizumab-associated PML.
Introduction

Progressive multifocal leukoencephalopathy (PML), which is caused by a reactivation of the John Cunningham Virus (JCV) is a serious complication in multiple sclerosis (MS) patients treated with natalizumab. Anti-JCV antibodies are associated with a higher risk of PML (1,2). It is hypothesized that this risk of developing PML is higher in patients with high anti-JCV antibody indexes in pre-PML samples (2-5). However, in studies presented so far, only very limited longitudinal samples were available. We here report four MS patients who developed PML during natalizumab treatment in whom extensive pre-PML samples were available.

Methods

Patients and materials

All relapsing-remitting (RR) MS patients treated with natalizumab in the MS Center Amsterdam, The Netherlands, were sampled before natalizumab initiation and at 3-monthly intervals. If applicable, samples were also taken at the time PML-immune reconstitution inflammatory syndrome (IRIS) was established and during plasmapheresis (PLEX) treatment. All serum samples were stored at -80ºC until shipped in one batch and assayed under masked conditions for clinical data at Unilabs, Denmark.

Anti-JCV antibodies were measured using the two-step second-generation JCV antibody ELISA, (STRATIFY JCV™DxSelect™), as previously described (3, 6). After the first step, serum was classified as negative (index <0.2), intermediate (index 0.20-0.40) or positive (index > 0.40). The second step, a confirmation test using pre-incubated samples with in-solution JC virus-like particles, was only performed in serum which showed an intermediate response. In the case of the percentage inhibition being ≤45%, serum was considered definitive negative, and if the percentage inhibition was >45%, it was considered definitive positive. Analytical sensitivities in monoclonal antibody equivalents are 60 ng/ml. We received quantitative results expressed as index values.

Four patients developed natalizumab-associated PML. The diagnosis was confirmed in three patients by detection of JCV DNA by quantitative polymerase chain reaction (qPCR) in the cerebrospinal fluid (CSF). In the other patient, MRI lesions were characteristic for PML, but no JCV DNA was detected by qPCR in the CSF. PML diagnosis
was further confirmed by clinical and MRI follow-up demonstrating a PML-immune reconstitution inflammatory syndrome (IRIS) five weeks after PLEX (for further details on PML diagnosis, see Wattjes et al.7).

This study was approved by the local institutional board (reference 2014.256). Informed consent was obtained from all participants.

Statistical analysis
Since the anti-JCV-antibody index was not normally distributed, log-transformation was performed. Subsequently, generalized estimating equations were used to investigate the association between anti-JCV antibody indexes and the presence of PML. Results are reported with their 95% confidence interval (CI). For continuous paired data, the Wilcoxon signed rank test was applied. The statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS, Inc., Chicago, IL, USA), with a statistical significance level of 0.05.

Results
In this group of 193 consecutive patients with RRMS who were treated with natalizumab, four patients developed PML. The mean age at first sample collection was 34.8 years old in the PML patients and 36.3 years in the non-PML patients. Two patients with PML (50%) were women vs. 131 (69%) in the non-PML patients. None of the PML patients used prior immunosuppressive medication.

In the four patients with PML serum anti-JCV antibody indexes were already high at baseline and subsequently in all available samples until PML diagnosis. (Figure 1(a) to (d)). From three patients, pre-natalizumab anti-JCV antibody indexes were available and already showed high indexes before start of treatment. The median anti-JCV antibody index before and during natalizumab therapy in these four patients was 3.04 with a range of 2.04-3.59. In all four patients, the anti-JCV antibody indexes had been relatively stable for years. Importantly, no meaningful increase was seen in the samples at the time of the diagnosis of PML compared with the baseline sample (median 3.05 vs. 3.16, p=0.72) or compared with the sample before diagnosis was made (median 3.05 vs 2.78, p=0.07).
From the 189 RRMS patients treated with natalizumab therapy who did not develop PML, we obtained 669 serum samples in which we measured the anti-JCV antibody index. These samples were obtained before and after the initiation of natalizumab treatment. Of the 176 patients in whom at least 2 samples were available, one of which after the start of natalizumab, 79 patients (44.9%) tested seronegative and 97 patients (55.1%) seropositive at the first moment of testing. The median index in the natalizumab treated MS patients who were positive at baseline and stayed positive or had no follow-up sample, but did not develop PML was 2,105 with a range of 0.260-3.941. The patients who developed PML had a 1.82 times (95% CI 1.51-2.19, p<0.001) higher anti-JCV antibody index compared with the patients without PML. Figure 1 also shows that at the moment PLEX was started, which was directly after the diagnosis, there is a decrease of the anti-JCV antibody index which increased again after the PLEX was stopped.

Discussion

We here describe four MS patients who developed PML during natalizumab treatment. From these four patients, a unique high number of pre-PML serum samples was longitudinally tested for anti-JCV antibody indexes. All four PML patients had consistently high serum anti-JCV antibody indexes in all samples obtained before the PML diagnosis, which is in line with earlier studies (2-5). In contrast to the patients described by Warnke et al.(2), overall no meaningful increase was seen in the anti-JCV antibody indexes in our four PML patients prior to the PML diagnosis. In the patients with a high anti-JCV antibody index (> 3.0), the explanation might be that high indexes technically cannot increase by reaching the saturation level of the assay (5).

Until now, known risk factors for PML have been treatment duration with natalizumab for more than two years, prior immunosuppressive therapy, and positive JCV status in serum. The duration of treatment of the four PML patients varied between 14 and 78 (mean 53) infusions. None of them had used immunosuppressive medication prior to natalizumab.

In this study we have shown that patients who developed natalizumab-associated PML had a consistently high anti-JCV antibody index which was even 1.82 times higher than in the JCV positive patients not developing PML. These findings confirm that the height
of anti-JCV antibody indexes in serum may have additional prognostic value for the risk of developing PML as was suggested in previous studies, in which limited longitudinal samples were obtained (2-5). In particular, Plavina et al. recently published that higher anti-JCV-antibody index, that is, > 1.5, is associated with a higher PML risk (5).

In addition, we here confirm that anti-JCV antibody indexes are decreasing during PLEX which was also described by Subramanyam et al (8). Furthermore, in line with data published by Ryschkewitsch and colleagues earlier (9), we also found that anti-JCV antibodies increased after the PLEX treatment was discontinued.

In conclusion, we here show that natalizumab-associated PML can be associated with rather stable high anti-JCV antibody indexes in pre-PML samples obtained long before PML diagnosis was established. Possibly caused by reaching the saturation level of the assay, an increase of the anti-JCV antibody index in pre-PML samples, as previously suggested, has not been observed in our patients. Our findings are in line with the recently published findings of Plavina et al. suggesting that higher anti-JCV antibody indexes are associated with a higher risk of PML.

Acknowledgements

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Figure 1: longitudinal anti-JCV antibody indexes of four natalizumab-associated progressive multifocal leukoencephalopathy (PML) patients. In all PML patients a relatively stable anti-John Cunningham Virus (JCV) antibody index is seen. In patient (c), a minor increase in index is seen just before diagnosing the PML. After the diagnosis, plasmapheresis (PLEX) is started immediately. At the moment PLEX is started, there is a decrease of the anti-JCV antibody index, which increases again after the PLEX is stopped. In this patients (c), multiple samples before initiation of natalizumab show that already more than a year before start of treatment a high anti-JCV antibody index is seen, which does not show a further increase after start of the natalizumab.
Start natalizumab

Diagnosis of PML

Median anti-JCV antibody index in MS patients without PML

Intravenous methylprednisolone 1000 mg for three days
Chapter 3.3

Application of serum natalizumab levels during plasma exchange in MS patients with progressive multifocal leukoencephalopathy

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Abstract
Progressive multifocal leukoencephalopathy (PML) is a severe complication of natalizumab treatment. Restoring immune function by plasmapheresis/immunoadsorption (PLEX/IA) is important for the outcome of PML. We report on four multiple sclerosis (MS) patients who developed PML during natalizumab treatment, in whom we measured serum natalizumab concentrations before and during PLEX. Depending on the serum natalizumab concentration at the time of PML diagnosis, the number of PLEX treatments necessary to reach subtherapeutic serum natalizumab concentrations is variable. Measuring serum natalizumab concentrations before and during PLEX is helpful to determine the optimum number of PLEX treatments in individual MS patients with PML.

Introduction
Natalizumab is a humanized monoclonal antibody that binds the cellular adhesion molecule α4-integrin. It blocks the migration of leucocytes into the central nervous system (CNS) and thus, reduces inflammation in patients with relapsing-remitting multiple sclerosis (RRMS). Progressive multifocal leukoencephalopathy (PML) is a severe complication of natalizumab treatment. The outcome of PML depends on the time to recovery of immune function after cessation of natalizumab-treatment. Plasmapheresis (PLEX) and immunoadsorption (IA) are used for accelerating the clearance of natalizumab from the body. Without intervention, natalizumab may still be measurable up to 200 days after cessation of therapy. Saturation of the natalizumab receptor is correlated with the serum natalizumab concentration: There is evidence that serum natalizumab levels should be < 1 μg/mL to achieve desaturation of the α4- integrin receptor to < 50%. In the majority of these patients, this can be achieved by five PLEX sessions, each of 1.5 plasma volumes, given 2 days apart.

This article describes the course of serum natalizumab concentrations, as measured by enzyme-linked immunosorbent assay (ELISA) during PLEX, in four RRMS patients whom developed PML during natalizumab treatment, suggesting that patients may benefit from natalizumab concentration-guided PLEX, as opposed to the standard regimen of five sessions.
Case reports

Case 1
A 40-year-old woman, diagnosed with RRMS in 2010, switched from interferon-β-1a to natalizumab in January 2012 because of clinical and radiological disease activity. In January 2013, a magnetic resonance imaging (MRI) scan that was made according to the local drug surveillance protocol (annually or every 3 months, if longer than 12 months on treatment and positive with positive JC virus serology) showed she had a new lesion in the subcortical white matter and adjacent cortical gray matter of the right frontal lobe, as well as multiple focal lesions having a perivascular pattern with partial gadolinium enhancement, highly suggestive of PML. Despite a negative JC virus deoxyribonucleic acid (DNA) quantitative polymerase chain reaction (qPCR) of the cerebrospinal fluid (CSF), a diagnosis of PML was made. She received her last natalizumab infusion on January 7 and started with five sessions of PLEX on January 25. As her natalizumab levels had always been relatively low, four PLEX sessions would have been sufficient to reach a serum natalizumab concentration below 1 μg/mL (Figure 1(a)).

Case 2
A 33-year-old woman was diagnosed with RRMS in 2000 and started interferon-β-1a the same year. Due to ongoing clinical disease activity, this therapy was switched to natalizumab in 2008. A routine MRI scan was made according to the local drug surveillance protocol, which showed a new vaguely-defined cortical and subcortical lesion in the right premotor cortex, and also less pronounced in the left, which was highly suggestive of PML. Quantitative PCR showed JC virus DNA copies in the CSF, fulfilling the criteria for probable PML. She received her last natalizumab infusion on March 28. On April 13, she started with PLEX. Retrospectively studying her serum natalizumab levels, it becomes clear that 10 days after the fifth PLEX, her serum natalizumab level was still in the therapeutic range (Figure 1(b)).

Case 3
A 41-year-old man was diagnosed with RRMS in 2003. From 2003 until 2008, he used interferon-β-1a. Because of clinical disease activity, he started with natalizumab treatment in 2008. Since starting the natalizumab, he had stable disease. He had MRI
scans every 3 months, because of positive JC virus serology. The July 2013 scan showed a focal lesion in the right occipital lobe, strongly indicative of PML. Positive JC virus DNA qPCR in the CSF led to a diagnosis of probable PML. He had his last natalizumab infusion on July 3. PLEX was started on July 18. After the fifth PLEX session, he still had a therapeutic level. For this reason, and given the experience of the previous case, we decided to perform two additional PLEX sessions (Figure 1(c)). In retrospect, if we would have measured the serum natalizumab level after the fifth PLEX, the additional PLEX treatments might not have been necessary.

Case 4
In 2003, in a 42-year-old man, the diagnosis RRMS was made. He used interferon-β-1a en interferon-β-1b from 2004 until 2007. Because of clinical activity, this therapy was then switched to natalizumab. The MRI scans were made according to the local drug surveillance protocol, the last years every three months, because of positive JC virus serology. In December 2013, the MRI scan showed a new large lesion in the middle cerebellar peduncle, on the left side and multifocal punctiform lesions on the rights side that were suggestive of PML. Quantitative PCR showed JC virus DNA copies in the CSF and a diagnosis of probable PML was made. He received his last natalizumab infusion on December 5 and PLEX was started on December 10. We measured his serum natalizumab concentration before and after every PLEX. After the seventh PLEX the serum natalizumab level was below 1 μg/mL (Figure 1(d)).

Discussion
Previous studies show that natalizumab concentration in MS patients is highly variable.\textsuperscript{7,8} Our report suggested that the number of PLEX sessions needed to reach natalizumab levels below 1 μg/mL depends on its level before PLEX. Patients with low serum natalizumab levels may not always need five PLEX treatments, whereas patients with high levels will sometimes need more. In the fourth case, we measured the serum natalizumab concentrations immediately after the PLEX treatments, using serum from before and after the PLEX treatment, so really concentration-guided decisions were made in this patient. Measuring serum natalizumab concentrations after the PLEX treatment seemed to be most helpful in deciding whether another PLEX treatment was going to be necessary or not.
We realize that instant natalizumab serum measurements are not available in all hospitals and countries worldwide. For the Netherlands, the service is covered by our central lab, Sanquin Diagnostic Services; however, we would like to emphasize that the test is relatively easy to implement.⁹

In conclusion, we believe that measuring serum natalizumab levels before and during PLEX may be helpful to tailor PLEX treatment to individual MS patients with PML. This will both prevent unnecessary treatment in some patients and inadequate premature termination of treatment in others. Although definite proof is lacking and more research is certainly needed, we hope and expect that this approach could lead to a better clinical outcome of natalizumab-associated PML in MS.
References


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Figure 1. Serum natalizumab concentrations before and during PLEX/IA treatment. (a) This patient always had low serum natalizumab concentrations (3.77 μg/mL before her 12th infusion). She had already reached a serum natalizumab concentration of 0.89 μg/mL (below therapeutic concentrations) before the fifth PLEX already. (b) In this
patient. The serum natalizumab concentrations were relatively high compared to the first patient, with still therapeutic concentrations before the fifth PLEX (4.60 μg/mL) and even 10 days later (2.67 μg/mL). (c) Also in this patient, the serum natalizumab concentration was in the therapeutic range (3.86 μg/mL) before the fifth PLEX. For this reason, and with the previous case in mind, we decided to give him two additional PLEX sessions. In retrospect, a week after the fifth PLEX session the serum natalizumab concentration had already dropped spontaneously below therapeutic concentrations. (d) This patient had a high serum natalizumab concentration before the start of the PLEX treatment (72.1 μg/mL). We measured serum natalizumab concentrations before and after every PLEX session. Eventually, after the seventh PLEX session, the serum natalizumab dropped below 1 μg/mL.

PLEX: plasmapheresis
Chapter 4
General discussion and future perspectives
Summarizing discussion and future perspectives

Introduction
In recent years much has changed in the treatment of relapsing-remitting multiple sclerosis (RRMS). Several new treatments became available and still more are coming up. However, with these new treatments, also “new” side effects were seen. Natalizumab is one of the most effective therapies on the market, but this effective treatment has a downside as well. Progressive multifocal leukoencephalopathy (PML), a severe, potentially live-threatening disease, caused by John Cunningham (JC) virus, is the most important reason not prescribing natalizumab to every patient with RRMS.

The purpose of this thesis was to learn more about clinically relevant issues of natalizumab treatment. Two questions had to be answered:
1. Which factors have a relevant influence on the effectiveness of the treatment?
2. Which are the most important risk factors contributing to the development of PML?

Further we will discuss how this knowledge may lead to a more individualized approach to natalizumab treatment in RRMS patients?

Natalizumab in the treatment of multiple sclerosis
In the first study (chapter 2.1), we investigated the clinical relevance of serum natalizumab concentrations and their relation with anti-natalizumab antibodies. It has already been shown that anti-drug antibody titers are much more difficult to standardize than serum drug concentrations. In a recent paper of van Schouwenburg et al., the difficulties of standardization of these anti-drug antibody measurements are highlighted and the authors conclude that it appears to be difficult to compare the results of different assays. The reason for this is that these results are highly dependent on the characteristics of the monoclonal antibody, most importantly the affinity of the antibody to the drug1.

In the initial phase 3 studies of natalizumab (AFFIRM and SENTINEL), it has been shown that 9-12% of the natalizumab-treated patients developed anti-natalizumab antibodies. In 6% it concerned persistent anti-natalizumab antibodies, which caused a loss of
efficacy as well as an increase in infusion-related reactions and the remaining patients had transient anti-natalizumab antibodies. Sorensen et al. found in their study 4.5% anti-natalizumab antibodies, 3.5% were persistent antibodies and Oliver et al. found in 14.1% of the patients anti-natalizumab antibodies of which 9.4 was persistent. In all these studies, the ELISA method was used.

In our study, anti-natalizumab antibodies were present in as many as 58% of the natalizumab-treated patients. At year one after start of the natalizumab, 95.4% of all patients of whom serum was available, was anti-natalizumab antibody negative. So, the total amount of patients with transient anti-natalizumab antibodies we found was much higher than in earlier studies. In our study, we used the RIA method. As was shown in a study of Hart et al., in which they studied the differential effect of drug interference in two common types of assays (RIA and ELISA), which were used to measure anti-adalimumab antibodies, the RIA method seems more suitable than the ELISA to detect anti-adalimumab antibodies with free adalimumab circulating in the serum as well.

We showed in one patient an increase in anti-natalizumab antibodies after cessation of the natalizumab therapy. This could suggest that anti-natalizumab antibodies are not measurable in the serum when these antibodies are bound to natalizumab and so called natalizumab-anti-natalizumab immune complexes are formed. The formation of these natalizumab-anti-natalizumab complexes also leads to an increase in clearance of natalizumab, and consequently results in a low serum natalizumab concentration. We could not demonstrate convincing clinical relevance of the measured transient antibodies in our patients. Most likely this is caused by the low antibody-titers, probably in combination with a low affinity, lacking to show a noteworthy effect on the natalizumab concentrations.

We found that the antibody titer was inversely correlated with serum natalizumab concentration (p<0.001) and that only high antibody titers were associated with very low or undetectable serum natalizumab concentrations. Both high antibody titers and low serum natalizumab concentrations were associated with clinical (relapses) and radiological (gadolinium-enhancing lesions on MRI) disease activity, indicating a lack of efficacy of natalizumab. We concluded that measuring natalizumab concentrations is as good as measuring anti-natalizumab antibodies for the evaluation of treatment efficacy. For various practical reasons, however, measurement of natalizumab concentration
could be preferable. Measuring natalizumab concentrations, using a highly specific assay, can also be a guidance in individualizing treatment strategies, which means that it will enable us to lower the dose or frequency of infusions in patients with a high serum natalizumab concentration.

An important consideration in this respect is that the amount of natalizumab infusions may contribute to the risk of PML and an increased interval between two infusions might have a positive effect on the risk of developing PML. This aspect has further been explored in the next chapter.

In the second study (chapter 2.2), we studied the effect of discontinuing the natalizumab treatment in 10 patients. In 7 out of these 10 patients there was clinical and/or radiological activity after discontinuation of treatment during a mean interval of 17 weeks (range 8 – 22 weeks). After discontinuing natalizumab, no other therapy was started. Also in other studies, it has been observed that discontinuing natalizumab treatment resulted in a return of disease activity and that this was independent of starting alternative therapies. These studies did not show a subsequent excessive disease activity, (“rebound”), after cessation of the natalizumab, while in some other studies they suggest it was. More studies have been done recently to further investigate the best strategy after natalizumab discontinuation. This will be further discussed in the future perspectives. As already mentioned before, the mean interval after which we found clinical or radiological disease activity was 17 weeks, with a range of 8-22 weeks. Khatri et al. described that after a single dose of natalizumab, it costs about 3 months to reach a natalizumab level <1 mg/l. A natalizumab level <1 mg/l would result in desaturation of the α4-integrin receptor to <50%. This was concluded after extrapolating natalizumab levels, which were monitored for 28 days.

To receive more insight in the course of natalizumab levels, which is among other things relevant in how to manage PML, we studied natalizumab levels in 10 RRMS patients who discontinued natalizumab therapy for different reasons (chapter 2.3). The mean treatment durations of these patients, before cessation of the natalizumab, was 23 months (range 12-40 months). We found that the median concentration in patients just before the last natalizumab infusion was 25 (range 18.4-104) mg/l. These concentrations already show that there is a wide range of natalizumab concentrations in the individual patients. Three months after the last natalizumab infusion the median
concentration was 0.28 (range 0.1-1) mg/l. There was still natalizumab detectable in some patients after more than 200 days. As we have shown in chapter 3.3, measuring natalizumab concentration is of major importance for individualized treatment in patients.

**Serological biomarkers and their relevance in natalizumab-associated PML in MS patients**

As already mentioned in the introduction of this chapter, natalizumab is a highly effective treatment for RRMS, but can be complicated by a rare, but potentially life-threatening adverse event, PML. PML is caused by the reactivation and replication of the JCV. JCV seropositivity is a risk factor for the development of PML. It is now known that patients with a high anti-JCV antibody index have a higher risk of developing PML than patients who are anti-JCV antibody negative or have a low anti-JCV antibody index. Nowadays, in the overall population the prevalence of anti-JCV antibodies is 57.1%-58.3% and tends to rise with increasing age\textsuperscript{11,12}. The incidence rates based on the estimated natural history are approximately 1%-2\%\textsuperscript{13}. Seroconversion rates in natalizumab-treated MS patients differed from 2%-26.7%\textsuperscript{14,15,16}. In all these studies there were limited longitudinal data, so the research question of chapter 3.1 was to evaluate the seroconversion rate in the natalizumab-treated RRMS patients of the VU medical center (VUmc). We found in our study population of 179 patients, with a median follow-up of 4.2 years (range 49 days to 12.7 years), an annualized seroconversion rate of 7.1%. This is in the same range as reported earlier by others, but with a much longer follow-up. If we take into account that RRMS patients will use natalizumab for many years, this would lead to a cumulative seroconversion rate of more than 25% in 4 years. This conversion rate is much higher than the estimated natural history conversion rate, and this fact should be taken into account in the risk assessment when considering the start of natalizumab treatment. At this moment, the European Medicines Agency (EMA) recommends retesting of the JCV-serology every 6 months during natalizumab therapy. The EMA decided last year to start an investigation, based on the reported seroconversion rate of 13% in 18 months by Plavina et al.\textsuperscript{15}, to reconsider the follow-up strategy of these JCV-seronegative patients. As we mentioned before, natalizumab-treated RRMS patients with a high anti-JCV antibody index have a higher risk than patients with a low anti-JCV antibody index. Plavina et al. provided in their paper a table
with estimated PML risk by anti-JCV antibody index and duration of natalizumab treatment\textsuperscript{15}. For example, a patient with more than 2 years of natalizumab treatment and an index of \textgreater{}1.5 has a risk of 8.1\% to develop PML versus 0.3\% in a patient with the same treatment duration, but with an index of \textless{}0.9\textsuperscript{15}. In earlier studies it has been suggested that an increase of anti-JCV antibody index could be observed in patients prior to the development of PML\textsuperscript{15,16,17,18}. The major limitation in all these studies was the very limited longitudinal samples that were available. That was the reason for us to further explore this aspect. This has been reported in the next chapter.

In chapter 3.2 the first 4 PML patients from the MS Center of the VUmc of whom extensive longitudinal pre-PML samples were available have been reported. Of 3 out of 4 PML patients, pre-natalizumab samples were also available. All these samples had already a high anti-JCV antibody index. In all samples of these four PML patients, the anti-JCV antibody indices were high, from baseline until the PML diagnosis. The median anti-JCV antibody index over time was 3.04 (range 2.04-3.59). These indices showed no significant increase or decrease over the years (median index baseline 3.05 compared to 3.16 at the time of PML diagnosis, $p=0.72$). The strength of our study was the exceptional high number of pre-PML serum samples in which we tested the anti-JCV antibody index. As mentioned before, all samples had already a high anti-JCV antibody index at baseline, which was also seen in the earlier mentioned studies\textsuperscript{15,16,17,18}. The difference with the patients described by Warnke et al.\textsuperscript{17} was that we did not see a significant increase of anti-JCV antibody index in our four PML patients preceding the PML diagnosis. There have to be taken into account that in patients with a high anti-JCV antibody index (above 3.0), the saturation level of the assay is already technically reached, so no increase of the index can be demonstrated anymore\textsuperscript{15}. The stable high anti-JCV antibody indices correspond to the earlier mentioned findings by Plavina et al.\textsuperscript{15}, that higher anti-JCV antibody indices are associated with a higher risk to develop PML.

One of the most important points of discussion for the future will be if we have to treat all patients the same. As we mentioned before, we think that measuring natalizumab levels could be of more value than measuring anti-natalizumab antibody titers (chapter 2.1), not only because measuring natalizumab levels is easier to standardize, but also because these levels can be used to individualize the treatment. For this purpose, we
studied the effect of plasmapheresis (PLEX) on natalizumab levels in 4 patients who developed PML. The results of this study have been reported in chapter 3.3. The intention of PLEX is to wash out the natalizumab and to restore the immune function. We found that the number of sessions needed to reach natalizumab levels below 1 mg/l (the level where desaturation of the α4-intergrin receptor to <50% is reached\textsuperscript{10}), depends on the natalizumab levels before starting the PLEX. This implicates that patients who have a low serum natalizumab level will not always need five PLEX sessions (standard procedure), whereas on the other hand patients with high serum natalizumab levels may need more than five PLEX sessions. This will lead to a more individualized treatment in natalizumab-associated PML and may prevent unnecessary PLEX sessions / premature terminating PLEX sessions in others.

**Future perspectives**

As mentioned already in the introduction, MS is the most frequent chronic disabling neurological disease in young adults. The frequency and severity of relapses partly determine the prognosis of persisting disability and progression to SPMS, which illustrates the relevance of early and effective treatment with as little as possible adverse effects for these patients.

Natalizumab is a highly effective treatment for RRMS\textsuperscript{19,20}. The risk of developing PML is the most important reason to refrain from or to discontinue natalizumab treatment. Plavina et al. developed a risk strategy which takes into account both the anti-JCV antibody index, prior use of immunosuppressive therapy, and the duration of natalizumab treatment\textsuperscript{15}.

However, we still would like to predict more precisely which patients using natalizumab are at risk of developing PML. Therefore future strategies to develop better biomarkers or a better combination of multiple biomarkers are still of major importance. Some research has been done with CD62L (L-selectin)\textsuperscript{21,22}. To determine CD62L, a laborious procedure with peripheral blood mononuclear cell (PBMC) samples is needed. Furthermore, CD62L has a lower sensitivity, but higher specificity than the anti-JCV antibody index in case of determining the risk of PML. Schwab et al. concluded that there might be an additional value of CD62L next to the anti-JCV antibody index in the future\textsuperscript{22}. In contrast, Lieberman et al. recently published a retrospective case-control study that
showed that CD62L was variable in serial sampling and the level was strongly influenced by the viability of the lymphocytes. For these reasons, CD62L seems not a predictive clinical biomarker for the development of PML\textsuperscript{23}. Further research on this and other biomarkers will be needed.

As shown in chapter 2.2, natalizumab discontinuation in RRMS patients can lead to severe clinical and radiological return of disease activity\textsuperscript{24}. More recently, some studies have been performed to reveal the best strategy how to deal with natalizumab discontinuation and how to find the best moment to start an alternative therapy. Iaffaldano et al. studied fingolimod versus interferon-\beta\textsubscript{1a}/glatiramer acetate after natalizumab discontinuation and found a superior effect of fingolimod\textsuperscript{25}. Cohen et al. showed that from the patients who switched from natalizumab to fingolimod 20% experienced a relapse in the first 6 months\textsuperscript{26}. The TOFINGO study showed the first evidence that a shorter interval (8-12 weeks) between the switch from natalizumab to fingolimod resulted in less clinical and radiological disease activity than after 16 weeks\textsuperscript{27}. Alping et al. described in an observational study that rituximab had a superior effect and tolerability compared to fingolimod after cessation of natalizumab\textsuperscript{28}.

Starting earlier with a new immunosuppressive drug after cessation of natalizumab might in theory give a greater risk to develop PML, but so far, in the TOFINGO study, no evidence was found that a shorter washout period of fingolimod increases this risk of developing PML. The occurrence of PML has been described in patients discontinuing natalizumab, which was concluded to be still a result of the preceding natalizumab therapy instead of a result of the newly introduced disease-modifying therapy (so called cross-over PML), although it was impossible to rule out any additional effect of steroids or this newly started therapy\textsuperscript{29,30}. The case described by Killestein et al. shows that PML-IRIS may still develop despite the lymphopenia induced by the fingolimod therapy\textsuperscript{30}. The effect of this lymphopenia is reversible in a short time. Considering the fact that some therapies such as alemtuzumab and rituximab result in depletion of circulating B- and T-lymphocytes for over a long period (approximately 6 and 12 months, respectively), this has to be taken into account when considering these therapies after the discontinuation of natalizumab. With the risk of still developing PML within the first months after discontinuation of the natalizumab, this might be a reason to first start, by example, fingolimod before considering a monoclonal antibody as alemtuzumab.
Breakthrough disease after discontinuation of natalizumab is still a problem for which no perfect solution is present, so this will be a major topic for future research.

New therapies are still coming up. Daclizumab high-yield process (HYP), a humanized monoclonal antibody directed against CD25, has been shown to be clinically and radiologically more effective than interferon-bêta in a phase 3 randomized, double-blind trial over 96 weeks (ARR 0.22 and 0.39, respectively and 54% lower amount of lesions with daclizumab HYP). Inherent to the better efficacy, more adverse events were observed, so the clinical benefit needs to be shown against the adverse events. Another new monoclonal antibody is ocrelizumab. Ocrelizumab, also a humanized monoclonal antibody that binds to CD20, was compared to interferon-bêta in the OPERA I and II study. The first presented results on the ECTRIMS 2015 showed that the patients treated with ocrelizumab experienced less relapses than the patients treated with interferon-bêta (0.15 and 0.29, respectively). Also less new and especially less gadolinium-enhancing lesions were seen in the patients treated with ocrelizumab. The most important adverse events were infusion-related events. So, the landscape of treatment for patients with relapsing remitting multiple sclerosis is still changing a lot nowadays. In the future we will learn if these new therapies will replace natalizumab therapy or if they are a good alternative after natalizumab discontinuation in case of high risk of PML.

Until now, natalizumab is one of the most effective therapies and we still have to find a way to treat patients with natalizumab in a way as safe as possible. One possibility, which might also lower the risk to develop PML, is lowering the dose or dose frequency of the natalizumab infusions. In the REFINE study, natalizumab 300 mg i.v. every 4 weeks was compared to 300 mg i.v. every 12 weeks, 300 mg s.c. every 4 and every 12 weeks and 150 mg i.v. and s.c. every 12 weeks. All study arms of every 12 weeks were stopped because of breakthrough disease was observed, which is not unexpected taking into account that (partial) desaturation of the natalizumab receptor will take about 100 days and clinical relapses after natalizumab cessation were seen after a mean interval of 17 weeks with a range of 8-22 weeks. Ryerson et al. retrospectively studied extended interval dosing and found that dosing intervals up to 8 weeks and 5 days did not diminish the clinical and radiological effect of natalizumab. The four PML patients in this study were all in the standard dosing (four weeks) patient group, but because of not
enough patient power of the extended dosing group, no statistically significant PML risk reduction can be achieved.

Foley et al. showed that serum natalizumab concentration and receptor saturation of natalizumab is inversely correlated with lower body weight, so patients with a low bodyweight have a higher natalizumab concentration in their blood as well as higher receptor saturation levels and is suggested to be a risk factor for PML\(^2^3\). As already mentioned before, we also found that individual patients have highly variable natalizumab levels\(^3^4\). Despite this knowledge, which will be caused by a large variation in pharmacokinetics and patient characteristics, all patients are still being treated with 300 mg natalizumab infusion every four weeks. We believe that almost 80% of the patients have natalizumab levels, which are much higher than needed to have an optimal clinical and radiological efficacy. In line with that, using natalizumab levels for dosing will result in less frequent natalizumab infusions and less hospital visits, which therewith will contribute to improvement in quality of life of RRMS patients. Additionally, there will be enormous financial benefits as well, which is approximately on annual base more than 2.5 million euro's in the Netherlands only.

Further research to prove that extending dose intervals based on measuring natalizumab levels will result in the same clinical and radiological efficacy and will lead to improvement of quality of life and significant cost-reduction is needed. Whether this personalized regimen will also decrease the risk of PML will be addressed as soon as extended dosing will be applied in large enough cohorts with long enough follow-up.

The landscape of therapeutic drugs available to treat MS continues to expand. As a result clinicians are faced with significant challenges in deciding the optimum treatment strategy for individual patients. A number of factors may influence this decision including JC virus antibody status, efficacy and potential adverse effects of alternate treatment, costs, regulatory and reimbursement issues. Even though quite some work has been done and in general, personalized medicine is practiced more widely now, a number of challenges still exist as mentioned above. Nevertheless, in my opinion a more personalized application of disease modifying therapies is the future, in particular for therapeutic monoclonal antibodies like natalizumab. I hope that this thesis will help to pave the road towards a more individualized therapy.
References


