

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 weeks 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 patients.

Keywords

Multiple sclerosis, natalizumab, natalizumab concentration, antibodies

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Introduction

Natalizumab is a humanized recombinant monoclonal antibody directed against very late active antigen (VLA)-4. Natalizumab binds to the $\alpha 4$ chain of $\alpha 4\beta 1$ integrin (VLA-4) and $\alpha 4\beta 7$ integrin and blocks the migration of leukocytes 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

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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 leukoencephalopathy, 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 -80°C until assayed at Landsteiner Laboratory Sanquin Research, 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.¹⁵ The detection limit of the assay is about 0.01 $\mu\text{g/ml}$. 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 $\alpha 4$ -integrin was observed to be less than 50% when natalizumab concentrations were below 1 $\mu\text{g/ml}$.¹⁶ Patients were therefore categorized as having low natalizumab concentrations if the natalizumab concentration was below 1.0 $\mu\text{g/ml}$.

Measurement of antibodies against natalizumab

Anti-natalizumab antibodies were measured with a newly developed RIA essentially following the protocol described by Bartelds et al.⁸ 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-idiotypic antibodies.¹⁷ 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 standard deviations (SD) measured in 100 healthy donors (data not shown). Based on analyses of antibody formation to adalimumab^{8,9} we distinguished between low anti-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 resolution of $1 \times 1 \text{ mm}^2$. 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 h 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 the 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 analyze 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 analyzed 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, VU University Medical Centre.

Four patients stopped natalizumab therapy prematurely during the first year of treatment. Three patients stopped due to side effects, two patients 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.

Table 1. Demographic and clinical characteristics.

N	73
Sex (% female)	80.8%
Age at start natalizumab (years)	37 (8.0)
Disease duration at start natalizumab (months)	112 (72)
Previous DMT last two years (n, %)	
Interferon-beta (IFN- β)	51 (69.9%)
Glatiramer-acetate (GA)	14 (19.2%)
IFN- β + GA	6 (8.2%)
Others	2 (2.7%)
Annualized relapse rate (ARR)	
Within 12 months before natalizumab	1.26 (0.93)
Within 24 months before natalizumab	1.48 (0.68)
EDSS at start natalizumab (median, IQR) ^a	4.0 (2.5)
0–3.5 (n, %)	26 (37.1%)
≥ 4.0 (n, %)	44 (62.9%)
Magnetic resonance imaging at start natalizumab (n, %)	
>9 T2 hyper intense lesions at baseline (n=73)	72 (98.6%)
Gadolinium enhancing lesions at baseline (n=71) ^b	45 (63.4%)
Follow up duration during natalizumab (n, %)	
<1 year	6 (8.2%)
1 year	36 (49.3%)
2 years	16 (21.9%)
3 years	15 (20.6%)

DMT: disease modifying therapy;

EDSS: Expanded Disability Status Scale; IQR: interquartile range.

^aEDSS at start natalizumab: 3/73 missing.

^bGadolinium at start natalizumab: 2/73 missing.

Data presented as mean (standard deviation (SD)) unless otherwise indicated.

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 antinatalizumab antibodies developed them before week 24. From all patients for whom serum was available at one year after the start of natalizumab therapy, the large majority of patients were 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 $\mu\text{g/ml}$). The patient who stopped because of clinical and radiological activity had very low natalizumab concentration (<0.001 – $0.10 \mu\text{g/ml}$) with very high anti-natalizumab antibody titers (range 80,000–260,000 AU/ml). Another patient

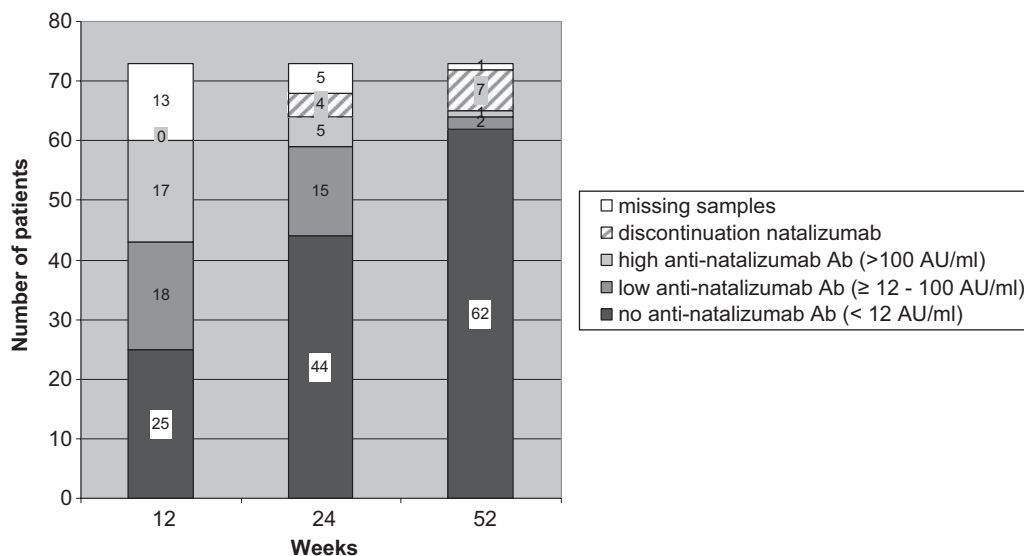


Figure 1. Antibody status during the first year. Available antibody status of all 73 patients at 12, 24 and 52 weeks (3, 6 and 13 infusions, respectively). It must be noted that of the seven patients who discontinued natalizumab treatment or were lost to follow-up, four patients had high antibody titers 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.

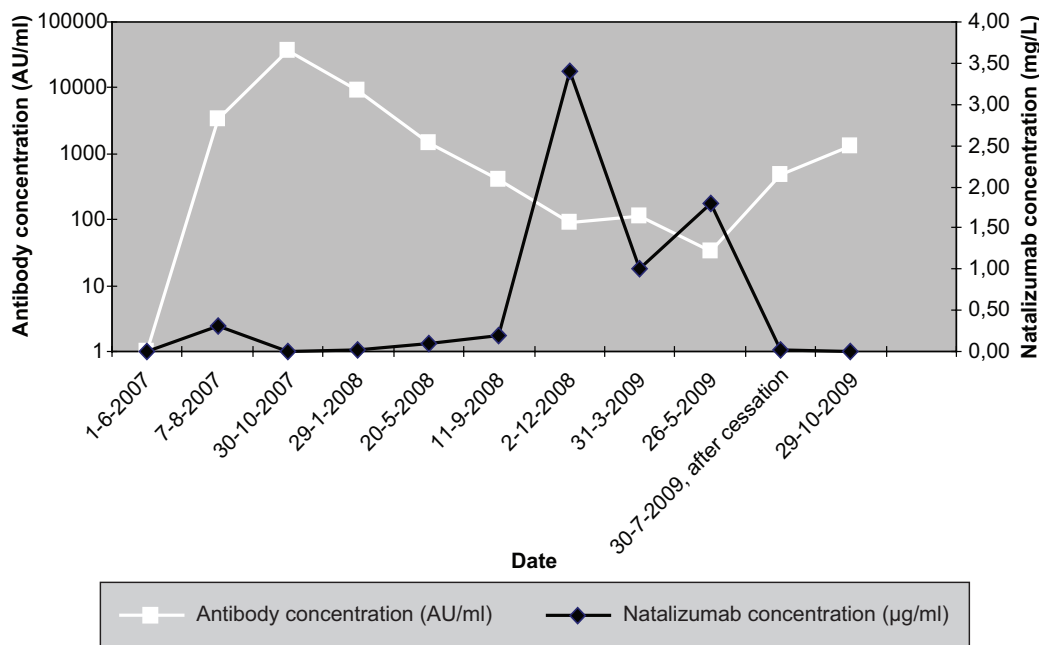


Figure 2. Anti-natalizumab antibody and serum natalizumab concentrations from one patient with high antibody titers during two years of natalizumab infusions and after discontinuation of the natalizumab infusions. The antibody titer was decreasing over the time and serum natalizumab concentration was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

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).

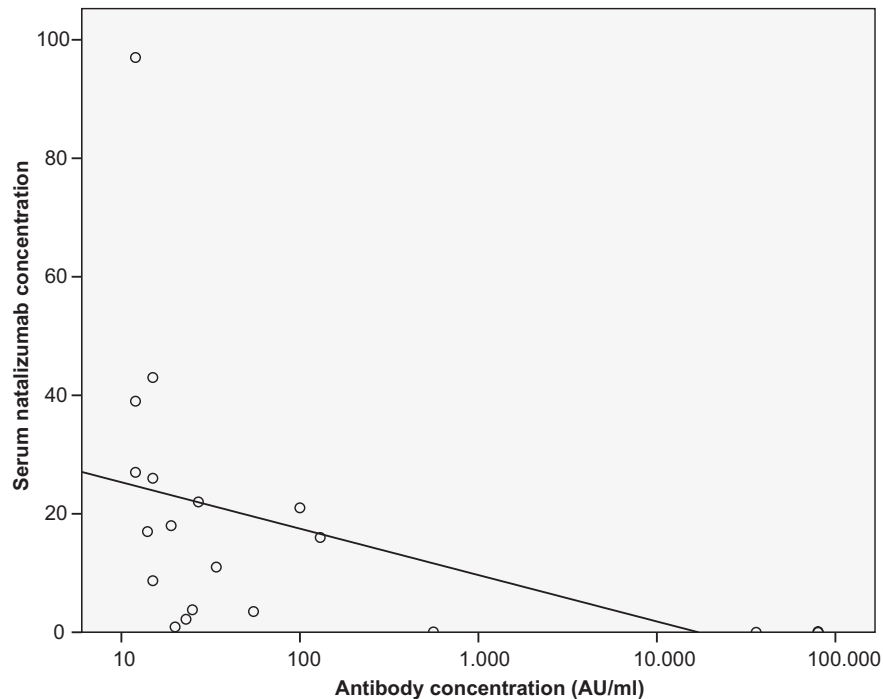


Figure 3. Scatter plot showing the correlation between anti-natalizumab antibodies and serum natalizumab concentration at week 24 (Spearman's Rho -0.765 , $p < 0.001$) in all patients with anti-natalizumab antibodies.

AU: arbitrary units.

Serum natalizumab concentration during natalizumab therapy

At week 12, median serum natalizumab concentration was 22 $\mu\text{g/ml}$ and varied from undetectable to 210 $\mu\text{g/ml}$. At week 24, median serum natalizumab concentration was 31 $\mu\text{g/ml}$ and varied from undetectable to 220 $\mu\text{g/ml}$. 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 (Figures 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 $< 1.0 \mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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 $\geq 1.0 \mu\text{g/ml}$ (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

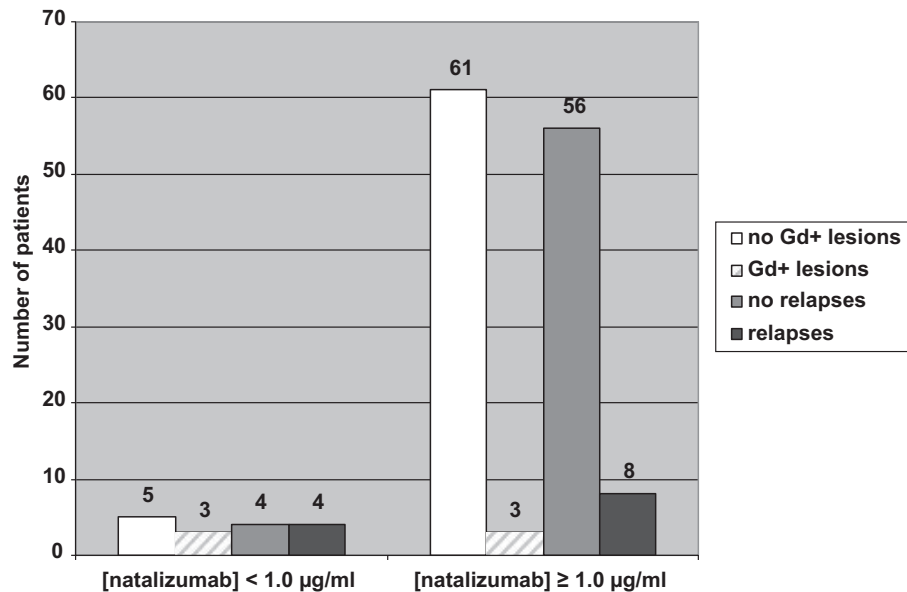


Figure 4. Low serum natalizumab concentrations have a 14.5 times higher odds ratio (OR) of 14.5 (95% confidence interval (CI) 2.2–96.4, $p=0.006$) to develop gadolinium positive (Gd+) lesions and a nine times higher odds (OR 9.0, 95% CI 1.7–47.9, $p=0.01$) to have a relapse compared to normal serum natalizumab concentrations.

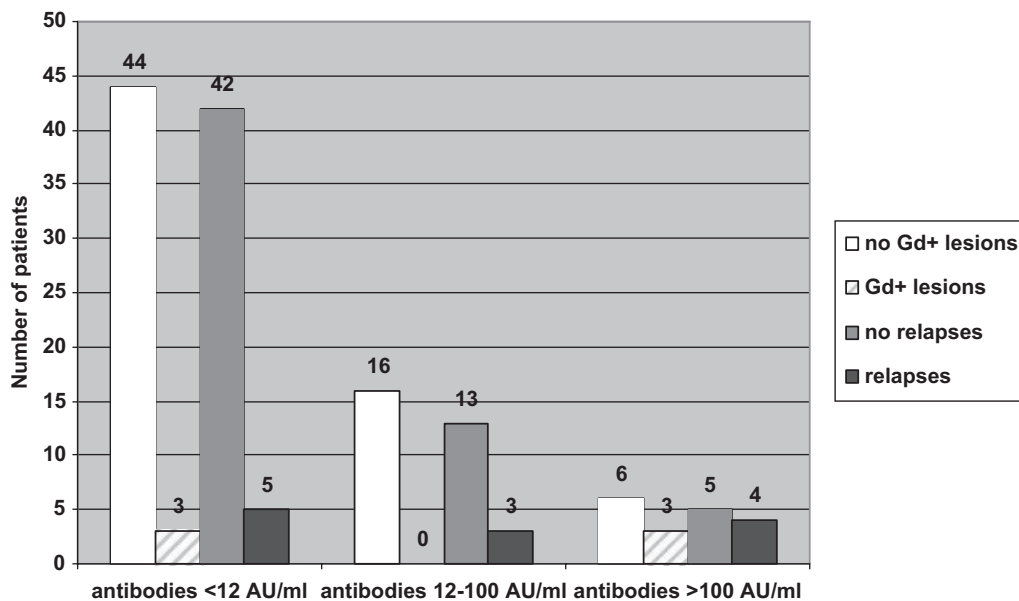


Figure 5. Patients with a high antibody titer have a 10.5 times higher odds ratio (OR) of 10.5 (95% confidence interval (CI) 1.6–70.3, $p=0.02$) to develop gadolinium positive (Gd+) lesions and a 10.9 times higher odds (OR 10.9, 95% CI 1.9–63.6, $p=0.008$) to have a relapse compared to patients with no antibodies.

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^{6–13} and have been reported previously for natalizumab to range between 4.5–14.1%.^{11–13} 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.¹⁸ 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 in 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-99m labelled infliximab.¹⁹ 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 of 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 for 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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Conflicts of interest

A Vennegoor: VU Medical Centre has received financial support for research activities from Bayer Schering Pharma, Biogen-Idec, GlaxoSmithKline, Merck Serono, Novartis and Teva.

T Rispens has received honoraria for lectures from Pfizer and financial support for research activities from Genmab.

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