

Wearing-off at the end of natalizumab dosing intervals is associated with low receptor occupancy

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Abstract

Objective

We aimed to investigate whether wearing-off symptoms at the end of the natalizumab dosing interval were associated with clinical and demographic patient characteristics or natalizumab receptor occupancy (RO) on leukocytes.

Methods

In this cross-sectional study of 40 patients with relapsing-remitting MS (RRMS) receiving natalizumab at the Department of Neurology, Haukeland University Hospital, we recorded clinical and demographic data including age, body mass index (BMI), working status, smoking habits, disease characteristics, treatment duration, vitamin D levels, and wearing-off symptoms. We quantified neurofilament light chain in serum and measured natalizumab RO in leukocyte subtypes by high-parameter mass cytometry. Associations with wearing-off symptoms were analyzed.

Results

Eight (20.0%) patients who reported regular occurrence of wearing-off symptoms, 9 (22.5%) who sometimes had wearing-off symptoms, and 23 (57.5%) who did not have wearing-off symptoms were evaluated. Patients who regularly had wearing-off symptoms had lower natalizumab RO than patients who reported having such symptoms sometimes or never. The former group also had higher BMI and higher frequency of sick leave. High BMI was associated with low RO. No other demographic or disease characteristics were associated with the phenomenon.

Conclusions

Low RO may explain the wearing-off phenomenon observed in some patients with RRMS treated with natalizumab, and high BMI may be the underlying cause.

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Glossary

BMI = body mass index; **cDC** = conventional dendritic cell; **EID** = extended interval dosing; **PBL** = peripheral blood leukocyte; **PML** = progressive multifocal leukoencephalopathy; **RRMS** = relapsing-remitting MS; **RO** = receptor occupancy.

Natalizumab (Tysabri[®], Biogen, Cambridge, MA) is a therapeutic monoclonal antibody used to treat patients with relapsing-remitting MS (RRMS). It prevents leukocyte migration across the blood-brain barrier into the CNS by binding to the $\alpha 4$ subunit of the $\alpha 4\beta 1$ integrin ($\alpha 4$ integrin) on leukocyte surfaces.¹ Natalizumab is administered IV at a standard dose of 300 mg every 4 weeks.

Although highly efficacious in preventing disease activity, many patients report the so-called wearing-off symptoms at the end of the 4-week dosing interval. Although wearing-off symptoms are often reported, only a few previous studies have described the phenomenon, and little is known about the underlying causes of these symptoms.^{2–5}

Natalizumab receptor occupancy (RO) is defined as the level of natalizumab bound to $\alpha 4$ integrin on leukocytes and is a potential biomarker to monitor and individualize natalizumab therapy.⁶ RO has traditionally been measured with flow cytometry. Mass cytometry is a novel technology for high-parameter single-cell analysis. For mass cytometry, detection antibodies are conjugated to metals instead of fluorophores, allowing analysis of over 40 parameters simultaneously on single cells.⁷ This permits measurement of RO in conjunction with more markers and in more cell types of interest than is currently possible by flow cytometry. We aimed to investigate whether clinical and demographic patient characteristics or natalizumab RO were associated with the wearing-off phenomenon by using high-parameter mass cytometry to measure natalizumab RO in patients with RRMS treated with natalizumab who do and do not report wearing-off symptoms at the end of dosing intervals.

Methods

Patients

We invited all patients older than 18 years with a diagnosis of RRMS who had received a minimum of 6 natalizumab infusions at the Department of Neurology, Haukeland University Hospital (n = 45) to participate in this cross-sectional study; 40 agreed to participate. At inclusion, we obtained baseline demographic and clinical patient characteristics from the patients' medical journal including age, sex, disease duration (years since first MS symptoms), natalizumab treatment duration (years since first natalizumab infusion), numbers of new MRI lesions and clinical relapses in the last year before inclusion, serum vitamin D level, Symbol Digit Modalities Test score,⁸ and Expanded Disability Status Scale score.⁹ Each patient filled in questionnaires on fatigue (Fatigue Severity Scale),¹⁰ and on working status, smoking habits, weight, height, and whether

they had wearing-off symptoms (never, sometimes, and regularly), and, if applicable, type of symptoms.

Standard protocol approvals, registrations, and patient consents

The study was approved by the Regional Committee for Medical Research Ethics, Western Norway (REK 2016/579), and written informed consent was obtained from all participating patients.

Blood samples

At inclusion, we collected blood before and after natalizumab infusion. For mass cytometry analysis, whole blood was collected in heparinized Vacutainer tubes (Greiner Bio-One GmbH, Kremsmünster, Austria), incubated with Proteomic Stabilizer (Smart Tube, Inc, San Carlos, CA) for 10 minutes, and stored at -80°C . Whole blood was then thawed, and red blood cell lysis was performed with Thaw-lyse buffer I (Smart Tube, Inc) to obtain peripheral blood leukocytes (PBLs). For neurofilament measurement, whole blood was collected in Vacutainer tubes with no additives (BD, Plymouth, United Kingdom), incubated at room temperature for 60 minutes, and centrifuged at room temperature at 3,200g for 13 minutes before the serum was retrieved and stored at -80°C .

Neurofilament measurement

Serum samples were thawed, and the concentration of neurofilament light chain (NF-L) was measured with a single-molecule array (Simoa) assay (Quanterix, Billerica, MA) according to the manufacturer's protocol.

Mass cytometry RO assay

PBLs were stained with a 36-parameter mass cytometry antibody panel (table e-1, links.lww.com/NXI/A190). Bound natalizumab was detected with an anti-IgG4 antibody (conjugated to ^{169}Tm). Total $\alpha 4$ integrin was detected with an anti-CD49d antibody (conjugated to ^{141}Pr) specific for a different epitope than natalizumab. The 36 metal-conjugated antibodies were purchased pre-conjugated (Fluidigm, South San Francisco, CA), or antibodies were purchased (BioLegend, San Diego, CA, R&D Systems, Minneapolis, MN, and Abcam, Cambridge, Great Britain) and conjugated in-house to metals with the Maxpar Antibody Labeling Kit (Fluidigm). Briefly, we thawed, barcoded (Cell-ID 20-Plex Pd Barcoding Kit, Fluidigm), and pooled PBL samples in batches of 20 randomly distributed samples, keeping paired samples from the same patients in the same batch. A control PBL sample from 1 healthy donor was included in each batch. Pooled PBLs were first incubated in Maxpar cell stain buffer with 100 U/mL heparin (LEO Pharma A/S) for 20 minutes at room temperature¹¹ and then incubated with the antibody cocktail

Table 1 Demographic and clinical characteristics of patients with RRMS and the frequency of wearing-off symptoms

	Wearing-off symptoms				p Value ^a	p Value (age adjusted) ^b
	Total	Never	Sometimes	Regularly		
Patients with RRMS, n (%)	40 (100)	23 (57.5)	9 (22.5)	8 (20.0)		
Age, y	43.0 (34.0–49.3)	45.0 (35.5–52.0)	34.0 (31.0–51.0)	43.0 (37.0–43.8)	0.382	—
Sex, female, n (%)	25 (62.5)	15 (65.2)	3 (33.3)	7 (87.5)	0.069	0.063
Height, cm	171 (166–179)	169 (165–173)	180 (177–182)	171 (167–173)	0.078	0.047
Weight, kg	75.0 (67.0–82.5)	75.0 (60.0–79.5)	73.0 (67.0–82.0)	81.5 (76.5–87.8)	0.107	0.022
BMI	25.3 (22.6–27.2)	25.1 (22.2–26.6)	23.9 (22.5–25.2)	27.8 (26.5–31.2)	0.023	0.006
Current smoker, n (%)	7 (17.5)	4 (17.4)	2 (22.2)	1 (12.5)	0.873	0.860
Sick leave, n (%)	9 (22.5)	2 (8.7)	3 (33.3)	4 (50.0)	0.04	0.013
Disease duration, y	13.0 (8.0–17.0)	13.0 (8.0–17.5)	13.0 (8.0–17.0)	12.5 (8.8–16.0)	0.949	0.798
Treatment duration, y	4.0 (3.0–7.3)	4.0 (3.0–8.5)	5.0 (3.0–6.0)	4.5 (2.0–8.0)	0.999	0.904
Dose number	56.5 (39.0–102.3)	49.0 (39.0–107.5)	69.0 (41.0–78.0)	63.5 (33.5–111.8)	0.974	0.816
Days since last dose	28.0 (28.0–28.0)	28.0 (28.0–28.5)	28.0 (28.0–28.0)	28.00 (27.0–28.0)	0.358	0.375
EDSS score	2.0 (1.0–3.5)	2.0 (1.0–3.5)	2.0 (1.5–2.5)	2.0 (1.5–3.1)	0.880	0.682
FSS score	4.8 (3.3–5.7)	4.6 (3.3–5.4)	4.3 (3.0–6.1)	5.3 (4.8–5.8)	0.223	0.186
SDMT	57.0 (50.0–67.3)	64.0 (52.5–68.5)	58.0 (54.0–64.0)	48.5 (45.5–56.0)	0.151	0.109
New lesions detected by MRI in last year	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	—	—
Relapse activity in last year	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	—	—
Serum vitamin D, nmol/L	74.50 (58.00–93.25)	76.0 (61.0–99.5)	74.0 (29.0–86.0)	73.0 (62.5–90.5)	0.796	0.773
Serum NF-L, pg/mL	6.1 (4.5–8.9)	8.0 (5.8–9.6)	5.1 (4.3–5.9)	4.6 (4.1–5.3)	0.011	0.390

Abbreviations: BMI = body mass index; EDSS = Expanded Disability Status Scale; FSS = Fatigue Severity Scale; SDMT = Symbol Digit Modalities Test; NF-L = neurofilament light chain. RRMS = relapsing-remitting MS.

Numbers are median (interquartile range) unless otherwise stated.

^a Unadjusted *p* values are calculated using a Kruskal-Wallis test.

^b Age-adjusted *p* values are calculated with a likelihood ratio test between a linear model with only age and a linear model with age and the relevant baseline variable as predictors.

for 30 minutes at room temperature. Stained PBLs were washed, fixed in fresh 2% paraformaldehyde (Thermo Scientific, Waltham, MA) in Maxpar PBS for 10 minutes at room temperature, and then incubated in 125 nM Cell-IDTM Intercalator-Ir in Maxpar Fix and Perm Buffer (Fluidigm) at 4°C overnight. We performed all centrifugation steps at room temperature at 800g.

Before mass cytometry analysis, PBLs were resuspended in 0.1× EQ Four Element Calibration Beads (Fluidigm) in Maxpar cell acquisition solution (Fluidigm) and filtered (Corning Falcon Test Tube with Cell Strainer Snap Cap,

Fisher Scientific, Hampton, NN). We used antibody binding QSC beads (Bangs Laboratories, Inc., catalog number 815A, Fishers, IN) to standardize signal intensities from anti-IgG4 (conjugated to ¹⁶⁹Tm) and anti-CD49d (conjugated to ¹⁴¹Pr) as previously described in detail.¹² We analyzed PBL and QSC beads with the same standard settings on a Helios[®] mass cytometer (Fluidigm) after tuning (CyTOF Tuning Solution, Fluidigm) and calibration (EQ Four Element Calibration Beads, Fluidigm) according to the manufacturer's guidelines. Healthy control PBLs served as a negative control for anti-IgG4 in the absence of natalizumab, and patient PBLs incubated in vitro with natalizumab to an expected RO of 100%

Table 2 Frequency of reported wearing-off symptoms

Symptom	Wearing-off symptoms	
	Sometimes	Regularly
Fatigue	67%	63%
Psychological	33%	25%
Walking difficulty	11%	25%
Spasms	11%	13%
Pain	0%	13%

served as positive controls. Further quality control experiments were performed as previously described in detail.¹²

Data processing, analysis, and statistics

After acquisition of QSC beads, we normalized (Fluidigm normalizer) and exported FCS files to Cytobank software (Cytobank, Inc., Beckman Coulter, Brea, CA) and created QSC bead standard curves (QuickCal template, Bangs Laboratories). After acquisition of PBL samples, we normalized (Fluidigm normalizer), debarcoded (Fluidigm Debarcoder), and exported the FCS files to Cytobank software for gating and downstream analysis (figure e-1, links.lww.com/NXI/A190). We performed clean-up gating to obtain single PBLs, and the data were arcsinh transformed with a scale argument of 5.¹³ We then performed 2 independent analyses for RO calculation in single PBLs: one was a manual analysis, and one used an unsupervised approach.

In the manual approach, we first identified the following 11 leukocyte subtypes of interest by manual gating: CD8⁺ central memory (T_{CM}), effector memory (T_{EM}), effector memory RA (T_{EMRA}) T cells; CD4⁺ T_{EM}, T_{CM}, and T_{EMRA} cells; CD34⁺ cells; memory B cells; natural killer (NK) cells; monocytes; and conventional dendritic cells (cDCs). We then plotted the signal intensities (dual count 90th percentiles) of anti-IgG4 and anti-CD49d in each of these cell types with the QSC bead standard curves to obtain bead standardized values and calculated %RO by the following formula:

$$\%RO = 100 \times \frac{\text{QSC bead standardized anti-IgG4 (169Tm)}}{\text{QSC bead standardized anti-CD49d (141Pr)}}$$

We compared ROs in the leukocyte subtypes in different patient groups using a Kruskal-Wallis test.

In the unsupervised approach, we used R (version 3.4.3) to add an extra variable into the FCS files: the ratio between signal intensities of anti-IgG4 and anti-CD49d in each cell. This resulted in an RO estimate for each cell. For visualization of high-dimensional single-cell data, we performed automated dimensionality reduction with stochastic neighborhood embedding (visNE, Cytobank).¹⁴ We analyzed the new RO variable with the cluster identification, characterization, and regression tool CITRUS (Cytobank), an algorithm that automatically

identifies statistically significant differences between patient groups.¹⁵ We applied the correlative model Significance Analysis of Microarrays with a false discovery rate (adjusted for multiple hypothesis testing) of 1%. CITRUS was run with 10 repetitions.

The relationship between baseline demographic variables and wearing-off was compared using a Kruskal-Wallis test. Statistical differences with $p < 0.05$ were considered significant using a 2-sided comparison. To test the age-corrected relationship between baseline variables and wearing-off, we used a likelihood ratio test between a linear model with only age and a linear model with age and the relevant baseline variable as predictors. We conducted a linear regression of the association between RO and body mass index (BMI) and used a t test to assess whether the slope was significantly different from zero. We used R version 3.4.3¹⁶ for statistical analysis and correlation plots.

Data availability

FCS files from anonymized patient PBL samples can be accessed in the Flow Repository (ID: FR-FCM-Z2A9).

Results

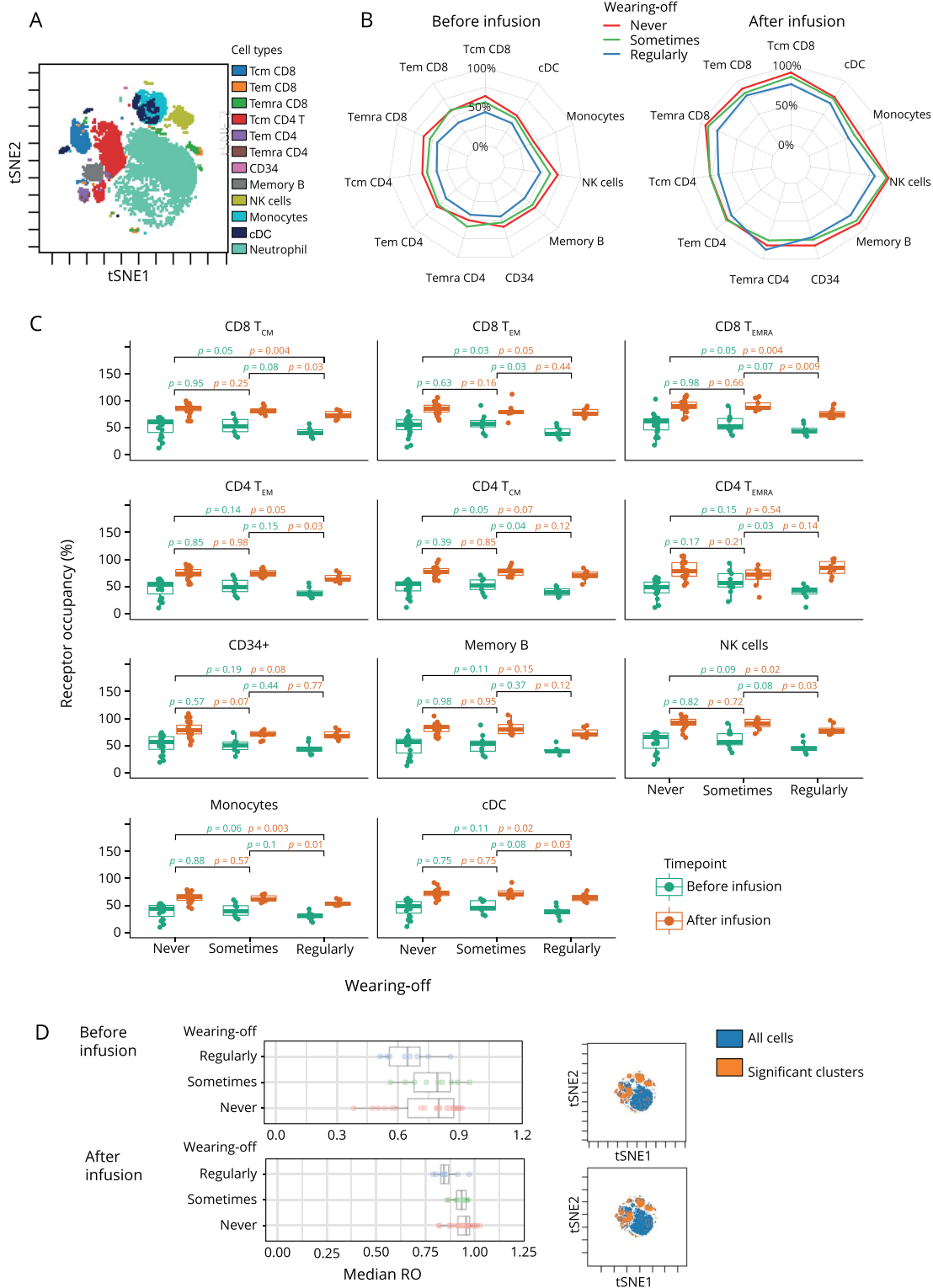
Patient characteristics

Of the 45 patients who were eligible for inclusion, 40 (89%) consented to participate in the study. Of 5 (11%) non-consenting patients, 1 refused participation, and 4 had infusion time points outside of the opening hours of the routine laboratory where blood samples were collected. Eight (20.0%) of the 40 participating patients reported having wearing-off symptoms regularly at the end of every dosing interval, 9 (22.5%) sometimes did, and 23 (57.5%) reported never having wearing-off symptoms (table 1). The most frequent wearing-off symptom was fatigue (table 2). Patients who regularly had wearing-off symptoms had significantly higher BMI and higher frequency of sick leave than patients who never or only sometimes experienced such symptoms (table 1). After age adjustment, weight was also significantly increased in patients with wearing-off symptoms regularly, whereas height was increased in patients with symptoms only sometimes. None of the other demographic or clinical patient characteristics were significantly different between the groups, and none of the included patients had clinical relapses or new lesions on MRI in the year before inclusion in the study. Age-adjusted median serum NF-L levels were similar between groups. There was no association between NF-L and BMI (data not shown).

Receptor occupancy

Manual gating of PBLs correlated well with the automated mapping with visNE (figure 1A). We observed a broad range of natalizumab RO values. The median RO values in all leukocyte subtypes before infusion and in 10 of 11 leukocyte subtypes after infusion (figure 1B) were lower in patients who regularly experienced wearing-off symptoms than in patients who never or only sometimes experienced such symptoms. The differences were statistically significant in CD8⁺ T_{EM}, CD4⁺ T_{EM}, and

Figure 1 Natalizumab receptor occupancy (RO) in patients reporting wearing-off symptoms never, sometimes, or regularly



(A) Manually gated PBLs visualized on a viSNE map. RO was analyzed in 11 cell subtypes: CD8⁺ central memory (T_{CM}), effector memory (T_{EM}), effector memory RA (T_{EMRA}) cells; CD4⁺ T_{EM}, T_{CM}, and T_{EMRA} cells; CD34⁺ cells; memory B cells; natural killer (NK) cells; monocytes; and conventional dendritic cells (cDCs). Neutrophils were not included in RO analysis. (B) Spider plot of median RO values in 11 cell subtypes in patients before and after natalizumab infusion. (C) RO values in 11 cell subtypes before and after natalizumab infusion. *p* values (Kruskal-Wallis test) comparing ROs in different wearing-off groups. (D) Left: median RO values in cell clusters significantly different between wearing-off groups (SAM analysis in CITRUS). Right: significant cell clusters are visualized on the viSNE map. PBL = peripheral blood leukocyte; SAM = Significance Analysis of Microarrays.

CD4⁺ T_{EMRA} cells before infusion and in CD8⁺ T_{CM}, CD8⁺ T_{EMRA}, CD4⁺ T_{CM} cells, NK cells, monocytes, and cDCs after infusion (figure 1C). Furthermore, in CD8⁺ and CD4⁺ T cells not stratified into subtypes, RO was significantly lower in both CD8⁺ and CD4⁺ T cells before infusion and in CD8⁺ T cells after infusion in patients who regularly had wearing-off symptoms (figure e-2, links.lww.com/NXI/A190). Unsupervised analysis of median ROs with CITRUS also showed lower median natalizumab ROs in patients with regular wearing-off symptoms (figure 1D). Neither of the 2 analysis approaches showed significant differences between patients reporting wearing-off symptoms sometimes and never.

High BMI was associated with significantly lower RO in CD8⁺ T_{EM} cells and cDCs before infusion and in CD8⁺ T_{EMRA} cells, CD34⁺ cells, and monocytes after infusion (figure 2).

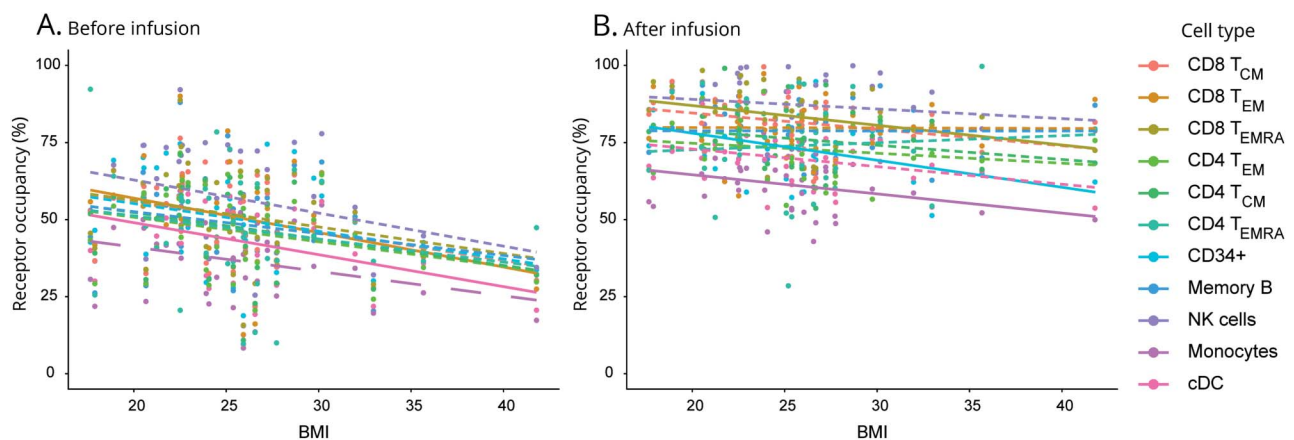
Discussion

Natalizumab prevents disease activity in RRMS and has positive effects on subjective symptoms such as mood, fatigue, and cognitive function.^{17,18} However, subjective wearing-off symptoms at the end of the 4-week interval are frequently reported by patients. In this study, wearing-off symptoms were reported by 42.5% of the patients (20.0% regularly and 22.5% only sometimes). We found lower natalizumab ROs in patients who regularly experienced wearing-off symptoms compared with patients who reported such symptoms never or only sometimes. The result was replicated in 2 separate data analysis pipelines. Furthermore, patients who reported regularly experiencing wearing-off symptoms had higher median body weight and BMI and higher sick leave frequency than those who rarely or never experienced such symptoms. Median height was increased in patients with symptoms only sometimes. Other clinical and demographic factors were

similar between the patient groups. High BMI was associated with low RO in several leukocyte subtypes.

The main limitation of our study is the small patient cohort. As a consequence of this limited statistical power, we were only able to detect large effects and acknowledge that there may be associations of smaller effect size that went unnoticed. Wearing-off symptoms were less frequent in our study than the prevalence of 54%–63% reported in other studies, but as previously reported, the most frequent wearing-off symptom was fatigue.^{2–5} In contrast to our results, a recent study found no association between the wearing-off effect and natalizumab RO or patient characteristics.⁵ The previously reported study used flow cytometry to measure RO in CD8⁺ T_{EM} cells and CD8⁺ effector T cells. By using high-parameter mass cytometry, we were able to measure RO in 11 cell subtypes simultaneously. We found that patients who regularly experienced wearing-off symptoms had higher BMI than those who did not, and we also observed an association between high BMI and low RO. Body weight was higher in our cohort than in the previous study⁵ (median 75.0 vs mean 72.9 kg) and was even more pronounced in the group with wearing-off symptoms regularly (median 81.5 vs mean 74.6 kg). van Kempen et al.⁵ reported nonsignificant trends similar to our significant results. Our wearing-off population had higher body weight than theirs, which may explain why we observed statistically significant differences despite our small cohort size. The association between high BMI and low RO suggests that high BMI, by decreasing natalizumab RO, may be the underlying cause of the wearing-off phenomenon. Others have previously reported such an association between low natalizumab RO and high body weight or BMI, and some have suggested that the dose of natalizumab should be adjusted for body weight.^{6,19,20} We found no associations between wearing-off symptoms and disease activity markers such as clinical relapses, new lesions detected by MRI in the past year, or serum NF-L levels.²¹

Figure 2 Linear regression analysis demonstrates an association between RO and BMI



Plot of receptor occupancies (A) before and (B) after infusion for indicated cell types as a function of BMI. Solid lines have slopes that are significantly different from zero ($p < 0.05$), and dashed lines have slopes that are not significantly different from zero. BMI = body mass index; RO = receptor occupancy.

As also reported by others,¹⁹ we observed large interindividual variations in natalizumab RO, and not all patients with low RO had high BMI. A previous study reported that body weight only partly predicts variability in natalizumab RO and suggested other factors such as density and turnover of $\alpha 4\beta 1$ integrin that may drive the variability.²²

It has been hypothesized that cytokines could induce the wearing-off symptoms.^{4,5} We speculate that lower natalizumab RO at the end of the dosing interval could increase the migratory capacity of cytokine-producing leukocytes into the CNS resulting in the wearing-off symptoms. We found significantly lower RO in the T-cell subtypes CD8⁺ T_{EM}, CD4⁺ T_{EM}, and CD4⁺ T_{EMRA} cells before infusion in patients who regularly had wearing-off symptoms. T_{EM} and T_{EMRA} cells home to the site of inflammation where they have effector functions such as secretion of proinflammatory cytokines and cytotoxicity; in contrast, T_{CM} cells and naive T cells home to secondary lymphoid organs.^{23,24}

Our results suggest that low RO may be a contributing factor to the wearing-off phenomenon and that higher BMI may be an underlying cause. This supports the suggestion that natalizumab dosing should be personalized. Personalization has been mainly focused on extended interval dosing (EID).¹⁹ This has particularly been driven by the risk of progressive multifocal leukoencephalopathy (PML), a serious complication of natalizumab therapy in patients previously exposed to JC virus.²⁵ Retrospective studies of off-label treatment with EID have shown maintained efficacy^{26,27} and reduced PML risk²⁸ compared with standard interval dosing. However, these studies are limited by possible selection bias due to nonrandomized design, and the efficacy and safety of EID is not fully known. As patients who regularly experience wearing-off symptoms already have lower RO and report symptoms at the end of dosing intervals, extending dosing intervals could increase the risk of disease activity. We therefore do not recommend EID in patients reporting wearing-off symptoms regularly, as this could lead to an even lower RO at the end of the interval than observed with standard dosing.^{6,19} Further studies should investigate whether wearing-off symptoms are associated with increased risk of RRMS disease activity and whether increasing RO by reduced dosing intervals or weight loss may mitigate the symptoms.

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Nello Blaser, PhD	University of Bergen, Bergen, Norway	Author	Interpretation of data; analysis of data; and revision of the manuscript for intellectual content
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Christian Alexander Vedeler, MD, PhD	University of Bergen, and Haukeland University Hospital, Bergen, Norway	Author	Conception and design of the study and revision of the manuscript for intellectual content
Sonia Gavasso, PhD	University of Bergen, and Haukeland University Hospital, Bergen, Norway	Author	Conception and design of the study; interpretation of data; and revision of the manuscript for intellectual content

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