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No evidence of JC-virus reactivation in natalizumab-treated Multiple Sclerosis patients: a 18-month follow-up.

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Abstract

Background and aim: Natalizumab, used as therapy for multiple sclerosis (MS), has been associated to progressive multifocal leukoencephalopathy (PML), a potentially fatal disease caused by JC virus (JCV), which is not predictable by specific markers. We evaluated whether JCV reactivation occurred in the urine and/or plasma, in 42 MS patients, treated with natalizumab over 18-months, and followed by a thorough monitoring program.

Methods: Forty-two patients (F/M: 24/18, mean age 34.4 ± 8.9 years) have been followed-up by: urine and plasma JCV-DNA PCR assay, immune cell subsets analysis, clinical and MRI evaluation, quality of life, fatigue and mood assessment.

Results: *JCV data.* At baseline 11/42 (26%) patients had JCV viruria, persistent at serial controls. One patient got viruric at month 1, one at month 12. No patient had JCV viraemia at baseline; 3 patients got viraemic: one at month 6, one at month 13 (both transiently), and one at month 12 (persistently viraemic). The prevalence of JCV both in urine and plasma did not change significantly from baseline to month 12 and 18. No patient had clinical and MRI evidence of PML. *Immunological data.* Circulating B cells showed the greater expansion (300% increase in absolute number) since the first infusion. NK cell count doubled with no change in percentage, while T cell count increased with a reduced percentage, reflecting a clear redistribution in the lymphocyte compartment. CD4+ and CD8+ T cells increased proportionally, with no change in their percentage. *Clinical data.* Twenty-seven patients (64%) were disease free after 1 year. A marked improvement in the quality of life was reported by 72 % of patients.

Conclusions: we found no evidence of subclinical JCV reactivation in our natalizumab treated MS patients up to the 18th month of therapy, notwithstanding the marked increase in circulating B cells observed. Moreover, we confirmed natalizumab efficacy, its tolerability, and the positive impact on quality of life.

INTRODUCTION

Natalizumab is the most recent drug approved for the treatment of relapsing-remitting multiple sclerosis (RRMS).¹ Natalizumab, a humanized monoclonal antibody, blocks the interaction between the integrin α_4 chain expressed by lymphocytes and the adhesion molecule VCAM-1 expressed by endothelial cells, thus preventing migration of lymphocytes into the central nervous system (CNS).²

Controlled trials showed the efficacy of natalizumab in reducing relapse-rate, disability progression, and lesion accumulation on magnetic resonance imaging (MRI) in RRMS patients.^{3,4} However, natalizumab therapy has unexpectedly been associated with a potentially fatal disease, progressive multifocal leucoencephalopathy (PML). Before the introduction of monoclonal antibodies, PML was mainly associated with HIV infection, chemotherapy, or immunosuppressive therapy, and was thought to be caused by the reactivation in immunocompromised patients of the polyomavirus JC (JCV), which is normally latent in the majority of healthy individuals.⁵⁻⁸ For this reason, in Europe natalizumab is currently approved as monotherapy only for RRMS patients with high disease activity despite treatment with other immunomodulating drugs, as well as for patients with a rapidly deteriorating disease. However, given the risk of PML (30 cases are currently reported), natalizumab is subject to specific international, European and national pharmacovigilance programs, calling clinicians for restricted criteria in patient selection, and an appropriate monitoring schedule.⁹⁻¹¹ Yet, we still do not know how to anticipate which patients will be at greatest risk of developing PML, and no reliable markers have been identified, based on which a specific monitoring program can be set up to recognize PML early. A recent pilot study describing a subclinical reactivation of the JCV in natalizumab-treated RRMS patients, showed the appearance of JCV DNA in urine, followed by the evidence of JCV DNA in circulating immune cells¹², thus suggesting that the detection of JCV in urine may predict a higher risk for PML.

Some attempts have also been done trying to explain the relationship between immunological changes, the clinical effects and the risk for PML in natalizumab treated RRMS patients. As expected by the expression of α_4 integrin on leukocytes, in the registration studies it was observed that natalizumab-treated patients had a reversible increase in leukocyte count, except neutrophils.^{3,4}¹³ Beside such effects, a number of other immunological alterations, not linked to the inhibition of lymphocyte migration into the CNS, have been described in natalizumab-treated patients. Among these, the mobilization of CD34⁺ hematopoietic precursors^{14,15} and B cell precursors¹⁶, in which latent JCV can initiate viral multiplication.

The purpose of this study was to evaluate whether JC virus reactivation occurred in the urine, plasma, or both in 42 RRMS patients, treated with natalizumab over an 18-month period, and followed by a thorough immunological, clinical and MRI monitoring program.

MATERIALS AND METHODS

Patients

Fourty-two consecutive patients (24 women and 18 men; mean age 34.4 ± 8.9 years, range 20-59; mean disease duration 10.3 ± 7.1 years (range 1-29); with a diagnosis of RRMS¹⁷ and regularly followed at the Multiple Sclerosis Centre of Veneto Region (Padova), were treated with natalizumab for at least one year, from July 2007 to October 2009. In the same period, a subgroup of 19 patients reached 18 months of treatment. All patients fulfilled the Italian Agency of Drug (AIFA) criteria for natalizumab treatment (Tysabri), i.e. not responsive to interferon (IFN) beta/glatiramer acetate (GA) (criterion A), or presenting an aggressive disease course at onset (criterion B). Thirty-eight patients (90.5%) fulfilled criterion A, having at least one relapse in the previous year while on therapy with immunomodulatory or immunosuppressive drugs (20 on IFN β -1a, 2 on IFN β -1b, 2 on GA, 9 on cyclophosphamide, 5 on mitoxantrone). Four patients (9%) fulfilled criterion B. The first administration of natalizumab was preceded by a “washout” period of at least one month for immunomodulatory, and six months for immunosuppressive drugs¹⁸. Patient mean Expanded Disability Status Scale (EDSS) score at T0 was 3.3 ± 1.2 (range 1-5.5), while the relapse rate during the twelve months before starting natalizumab was 2.1 ± 0.8 (Table 1). The study was approved by the local Ethic Committee.

Clinical follow-up

At baseline all patients underwent a complete physical and neurological examination. Neurological disability was assessed by the EDSS¹⁹. Based on the accurate surveillance program indicated by AIFA, all patients underwent a clinical follow up consisting of trimestrial EDSS score assessment and record of side effects and adverse events. Patients filled in a questionnaire for the most common immediate and delayed hypersensitivity reactions to natalizumab (i.e. itch, cutaneous rash, fever, arthralgia, headache, fatigue). The side effects frequency was reported according to the World Health Organization recommendations (very common, > 10%; common, >1% and < 10%; uncommon, > 0.1% and < 1%; rare, > 0.01% and < 0.1%; very rare, < 0.01%).²⁰

Detection of Viral DNA by qualitative PCR assay

JCV presence was assessed in urine and plasma by qualitative real-time polymerase-chain reaction (PCR) at baseline, after 1 month, and after 6, 12, 13 and 18 months of natalizumab therapy. Nucleic acid was extracted from 250 μ l of each specimen (urine and plasma) using the BioRobot 9604 workstation (Qiagen) and the QIAamp Virus BioRobot Kit (Qiagen) according to the manufacture's protocol. The DNA was eluted in 100 μ l. Each real-time PCR mixture consisted of 25 μ l Taqman universal PCR master mix (Applied Biosystems, Foster City, CA, USA) 600 nM TaQ primers, 250 nM JC-P Taqman probe (Table 2), 17 μ l water PCR grade and 5 μ l of extracted

sample. Thermal conditions were 95° C for 10 minutes followed by 45 cycles at 95° C for 15 seconds, 60° C for 1 minute. Reactions were amplified in a 7900HT Sequence Detection System (Applied Biosystems). In case of positive assay, a quantitative analysis was performed with a detection limit of 42 DNA copies/ml.

Laboratory and viral follow-up

Laboratory tests included complete blood count and differential, aspartate and alanine aminotransferase, γ GT, total bilirubin, Herpes Simplex virus (HSV) 1 and 2, Cytomegalovirus (CMV) and Toxoplasma Gondii antibodies. Complete blood count and liver function were carried out monthly, while HSV 1 and 2, CMV and Toxoplasma Gondii antibodies every 12 months.

Immunological follow-up

For the prospective flow cytometry analysis of blood immune cell subsets, whole blood direct staining was performed using a standard procedure.²¹ Stained cells were analysed on a two-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems, San Jose, CA), using Cell Quest and DIVA softwares (BD Biosciences) for data acquisition and analysis, respectively. Reported percentage values are referred to the parental lymphocyte population. The absolute count of each lymphocyte subsets was calculated based on the percentage and the differential WBC. The following mAbs were used: CD45 FITC (pan-leukocyte antigen), CD3 PerCP (T lymphocytes), CD4 PE-Cy5 (CD4⁺ T cells), CD8 PE (CD8⁺ T cells), CD16 FITC (Natural Killer, NK Lymphocytes), CD16 PE (NK Lymphocytes), CD19 PE (B lymphocytes), CD56 PE (NK Lymphocytes) (all purchased from BD Biosciences).

Neuropsychological follow-up

Cognitive functions were tested using the Rao's Brief Repeatable Battery (BRB) of Neuropsychological Test, version A;²² quality of life, fatigue and mood were evaluated by Multiple Sclerosis Quality Of Life-54 scale (MSQoL-54), Fatigue Impact Scale (FIS), Fatigue Severity Scale (FSS) and Beck Depression Inventory (BDI) scale. MSQoL-54, FIS, FSS and BDI scale were re-administered one year after therapy start, whereas cognitive evaluation after two years.

MRI follow-up

Cerebral MRI was performed on all patients at baseline and after 12 months of natalizumab therapy, using the protocol for image acquisition currently used in our Centre.²³

Statistical analysis

All data were prospectively collected, using pre-treatment values as referral. Statistical analysis was performed using GraphPad InStat Software and SPSS. The prevalence of JC virus detected in urine and plasma at each time point (baseline, month 1, 6, 12, 13 and 18) was compared with the use of a two-sided Fisher's exact test. The absolute count and percentage of the immune cell subsets

were compared to the baseline values at each month, using a repeated measures one-way ANOVA, with post test (Dunnett) as correction for multiple comparisons. A general linear model for repeated measures was used to compare immune cell subset changes during the follow-up between natalizumab treated and untreated MS patients (control group). Wilcoxon matched-pairs signed-ranks test was used to compare the clinical variables at baseline and after one year of natalizumab treatment. Differences were considered significant if $p < 0.05$.

RESULTS

Clinical outcomes

In October 2009, 42 out of 70 RRMS patients (60%) currently on natalizumab therapy at our Centre, reached at least one-year follow-up (19 of which got to 18 months). In these patients the mean EDSS score remained stable (3.2 ± 1.3 , range 1-6.0) after twelve months of natalizumab, being 3.3 ± 1.2 , (range 1-5.5) at baseline. Individual EDSS score did not change in 15 patients (35.7%), decreased in 15 (35.7%), and increased (not more than 0.5 points) in the remaining 12 (28.6%). Thirty patients (71.4%) were free from disability progression after twelve months of treatment. Thirty-nine patients (92.8%) remained relapse-free from baseline to month 12, with a remarkable reduction in the mean relapse rate compared to the previous year (from 2.1 ± 0.8 to 0.09 ± 0.3 ; $p < 0.0001$) (Table 1). Nineteen patients showed a similar clinical trend (data not shown). We observed only three bouts of mild intensity, occurred some days after the first natalizumab infusion in two patients and after eight infusions in one case: all relapses were successfully treated with high-dose methylprednisolone and all patients could continue natalizumab. Collectively, 27 patients (64%) were disease free after 1 year of natalizumab therapy.²⁴

Common side effects and laboratory abnormalities in our patient cohort are reported in Table 3. Notably, one patient presented a first generalized epileptic fit, after the 10th natalizumab infusion: once ruled out PML by brain MRI and CSF search for JCV DNA, this patient started anti-epileptic drugs, and continued natalizumab.

JCV outcomes

Detection of Viral DNA in Urine

In our cohort of 42 RRMS patients, we observed that all patients who were positive to JCV in urine at baseline, remained persistently positive throughout the follow-up (i.e., at all time points tested, month 1, 6, 12, 13, 18). The prevalence of JCV in urine was 26% at baseline (11/42) and did not change significantly from baseline to month 12 (13/42, 31%, two-sided Fisher's exact test, $p = 0.49$), and to month 18 (3/19 patients who have reached this time point at present, two-sided Fisher's exact test, $p = 0.52$; Table 4).

Detection of Viral DNA in Plasma

In our RRMS cohort, no patient was JCV positive in plasma at baseline. Three patients, however, got plasma JCV positive between baseline and month 18. Two of them were only transiently JCV positive in plasma (one at month 6, the other at month 13), and both patients got negative within one month after the last natalizumab infusion, without getting plasma JCV positive any more till month 18. The third patient got JCV positive in plasma at month 12, and remained persistently positive in consecutive plasma samples, prompting precautionary therapy discontinuation. This patient did not develop any sign or symptom suggestive of PML up to now. All the three patients who converted to JCV in plasma were JCV positive in urine since the baseline (Table 4), and fulfilled the criterion A for natalizumab treatment: the two patients who got transiently plasma JCV positive were previously treated with interferon β and immunosuppressors, the third one only with immunomodulatory drugs.

PML

None of our natalizumab-treated patients followed for 12 (42 patients) and 18 months (19 patients), showed either a worsening of the clinical picture suggestive of PML, or MRI findings consistent with this diagnosis, including the patient with a first epileptic seizure, whose CSF resulted JCV DNA negative.

Other microbiological outcomes

The analysis of antibodies (IgM and IgG) against HSV1-2, CMV and Toxoplasma Gondii provided no evidence for an altered immune response toward these agents.

Immunological outcomes

We observed that all the changes in circulating immune cells occurred since the first administration of natalizumab and persisted through the year. Such changes were natalizumab related as shown by the comparison with a cohort of untreated MS patients.²¹ Except for the B cell percentage, the mean values of all the other circulating immune cells analysed remained within normal ranges (Table 5).

Leucocyte subsets

The leukocyte count significantly rose since the first natalizumab administration ($p=0.0003$), while it remained stable in the control group, as well as the absolute count and the percentage of all the leukocyte subsets. On the contrary, in natalizumab treated patients we observed a prompt and marked elevation of the lymphocyte ($\Delta=+100\%$, $p<0.0001$) and eosinophil count ($\Delta=+180\%$, $p=0.0051$), accompanied by a rise of their percentage ($\Delta=+40\%$ and $+100\%$, $p<0.0001$ and $p=0.0149$, respectively), and accounting for the increased leukocyte count observed. The monocyte

count showed only a trend of increase, while the neutrophil and basophil count did not significantly change throughout the treatment.

B lymphocytes dramatically increase during natalizumab therapy

Among circulating lymphocytes, the most dramatic increase was observed in B cells, which promptly tripled after the first natalizumab dose ($\Delta=+250\%$, $p<0.0001$), with a corresponding increased percentage ($\Delta=+110\%$, $p=0.0001$, Fig.1). This highly significant rise of B cells was persistent and natalizumab related, as demonstrated by the comparison with the control group ($p<0.0001$ by general linear model analysis, Fig. 2). Though less conspicuous, NK cell count increased as well ($\Delta=+110\%$, $p=0.0008$), while their percentage remained unchanged. T lymphocyte count showed the less remarkable increase ($\Delta=+70\%$, $p<0.0001$) during natalizumab treatment, thus accounting for the T cell percentage decrease observed ($\Delta=-13\%$, $p=0.0002$). Overall, these data show that natalizumab does not simply cause a lymphocytosis, but it determines a clear redistribution in the lymphocyte compartment, differentially affecting T, B and NK cells, in favour of B cells.

CD4⁺ and CD8⁺ T cells

Interestingly, while the count of both CD4⁺ and CD8⁺ T cells increased ($\Delta=+80\%$ and $+90\%$, $p<0.0001$ and $p=0.0073$, respectively), their percentage remained stable, as well as CD4/CD8 ratio, showing that these T cell subsets are proportionally affected by natalizumab. NK-like (CD3⁺CD16⁺CD56⁺) T cells did not show any significant change during Tysabri treatment.

Quality of life and fatigue

Thirty-nine patients filled in the MSQoL-54: 28 (72%) reported an improvement in their quality of life (≥ 5 points of score increase), while only 6 patients (15%) had a worsening of their quality of life. The mean MSQoL-54 score at 12 months was significantly higher, compared to baseline, in both the physical health composite score (70.6 ± 14.5 vs 57.2 ± 19.0 , $p<0.001$) and the mental health composite score (72.1 ± 16.2 vs 58.4 ± 19.6 , $p<0.001$). Moreover, we observed a significant fatigue decrease in 17 out of 35 patients (49%) who filled in the FIS questionnaire, despite the marked state of exhaustion frequently reported the day after natalizumab infusion. Only 9 (26%) patients reported a worsening of fatigue (≥ 10 point increase in FIS score). Finally, we did not observe a significant change in patient mood after one year of natalizumab therapy.

MRI outcomes

Brain MRI scans were available for all our patients at month 12. The T2 lesion volume remained stable in 40 patients (95.2%); one patient developed a new supratentorial T2 lesion, while another patient developed two new supratentorial lesions, all typical for MS. Only one patient (2.4%) had a new Gd⁺ lesion at follow-up.

DISCUSSION

Natalizumab represents a major breakthrough in the treatment of MS, a chronic inflammatory disease of the CNS, which can cause a relevant disability in young adult patients. Indeed, the data from our cohort of 42 MS patients strongly confirm the efficacy of natalizumab in controlling the inflammatory component of MS: 64% of our patients resulted disease-free after 1 year of natalizumab treatment, with no disability progression, a marked reduction in the relapse rate, and no evidence of burden increase on brain MRI. Moreover, the majority of our patients (72%) reported a significant improvement in their quality of life, as well as a decrease in fatigue. Besides its efficacy, we observed that natalizumab was generally well tolerated, compared to other current therapeutic regimes, and no major side effects were registered during the 18-month follow-up.

However, natalizumab therapy has been associated with PML, a potentially fatal disease which was never described in MS patients before the introduction of monoclonal antibodies as immunomodulatory treatment. PML is a rare, opportunistic, demyelinating viral infection of the CNS caused by JCV, and normally affecting immunocompromised patients (HIV, chemotherapy, immunosuppressive therapy). At January the 25th 2010, 30 cases of PML in natalizumab-treated MS patients have been confirmed by FDA and EMEA, 22 as monotherapy, with a calculated risk of about 1:1000. Such a risk raises some concerns, due to the high mortality rate of this disease, even though two recent reports have described an effective therapeutic protocol for the treatment of PML. Moreover, the mechanism by which natalizumab can cause PML is still not known, and there are no reliable predictive markers of disease. Indeed, conflicting opinions have been expressed on the predictive value of JCV viraemia for the risk of PML^{12 25-28}, and no consensus exists yet in recommending the use of serial testing for blood and urine JCV DNA. In this scenario, it is of paramount importance to assess the protocols of pharmacovigilance proposed for the prediction and early recognition of PML. Thus, we decided to include urine and plasma JCV DNA determination in our monitoring schedule of natalizumab-treated patients, to evaluate whether and when a subclinical reactivation of JCV occurs during treatment, and whether this may precede the development of PML.

In our cohort of 42 RRMS patients, the prevalence of JCV in urine at baseline was 26%, similar to that previously reported in normal subjects, as well as MS and Crohn's patients before natalizumab treatment.^{12 28-31} During the therapy follow-up no significant increase in this prevalence was observed at 12 and 18 months. Moreover, while no patient had JCV in plasma at baseline, the occurrence of two transient and one persistent JCV viraemia, did not significantly change JCV prevalence. Indeed, neither any patient developed PML, nor did we find any evidence of subclinical JCV reactivation in our patients after 18 months of natalizumab therapy.

Our findings are in contrast with a recent pilot study, in which a subclinical JCV reactivation was described in a consistent proportion of 19 natalizumab-treated MS patients.¹² After 12 months of treatment, the Authors observed a significant increase (300%) in the prevalence of JCV in urine, followed, after 18 months, by an increase of JCV in PBMC. None of the patients in that study developed PML. Whether a subclinical JCV reactivation may herald the development of PML could not be determined. However, in a recently described case of PML, the absence of JCV in plasma would question such an occurrence, and even the absence of JCV in CSF can not exclude an ongoing PML, due to the modest sensitivity (60-80%) of the test.³² Generally, in the MS patients who developed PML and whose data are available, JCV was undetectable both in plasma and PBMC, despite its positivity in CSF.^{7 8} Moreover, in a very recent report on 200 natalizumab-treated patients, JCV DNA was detected only in 1,5% of the patients, either in plasma or CSF. Notably, all patients converted to undetectable viral DNA after cessation of therapy, and no patient had clinical evidence of PML.³³

Another crucial aspect in the monitoring of JCV is the potential host reservoir of this widespread virus.³⁴ Apart from the kidney, JCV can be detected in peripheral blood lymphocytes, particularly B cells, as well as in B-cell precursors from the bone marrow.³⁵⁻³⁷ Interestingly, a release of B cells and B-cell progenitors has been shown in patients receiving natalizumab^{10 15}, and our findings of a prompt and marked, natalizumab-dependent increase in the circulating B cells of all patients are strongly consistent with these recent studies. However, despite this dramatic phenomenon, likely due to the effect of natalizumab on B cell precursors expressing VLA-4 in bone marrow³⁸, we had no evidence that this event resulted in JC viraemia in our patients.

In conclusion, despite the limits of this study (low number of patients followed up to 18 months or further, and no JCV DNA analysis in PBMCs), our data confirm the overall efficacy and good safety profile of natalizumab therapy, and show that transient shedding of live JCV may occur not only in urine, but also in plasma, with no apparent pathogenic relevance. While our and others' findings do not support urine JCV determination as a valuable tool for natalizumab-treated patients follow-up, the persistent positivity of JCV DNA in plasma or PBMCs as predictor of PML deserves further evaluation. Indeed, waiting for a consensus and new guidelines in this regard, we and others propose to precautionary discontinue therapy in case of persistent JCV plasma positivity. Extending the investigation on virological and immunological effects of natalizumab will be essential for a better management of this drug in MS patients.

Figure Legend

Figure 1: Diagram representing the longitudinal distribution of B cell count and percentage in the natalizumab treated and a reference group of untreated MS patients followed for one year: in treated patients B cells markedly increase since the first natalizumab administration, while in untreated patients B cells clearly remain stable over time, thus demonstrating that the B cell rise in treated patients is driven by natalizumab. Dots represent the mean values of each time point, plus SD.

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Table 1. Demographic and clinical data of patient population (*n*=42).

Gender	
Female (<i>n</i> , %)	24 (57%)
Age ^a (mean, SD)	34.4±8.9
(range)	(20-59)
Disease duration (years) ^a (mean, SD)	10.3±7.1
(range)	(1-29)
EDSS at baseline (mean, SD)	3.3±1.2
0-1.5 (<i>n</i> , %)	3 (7%)
2-2.5	16 (38%)
3-3.5	6 (14%)
4-4.5	10 (24%)
5-5.5	7 (17%)
EDSS at month 18 (mean, SD)	3.2±1.3
0-1.5 (<i>n</i> , %)	7 (17%)
2-2.5	12 (28%)
3-3.5	7 (17%)
4-4.5	10 (24%)
5-6	6 (14%)
Annualized relapse rate	
Previous 12 months	2.1±0.8
Next 12 months	0.09±0.3 ****
Previous DMT	
None (<i>n</i> , %)	4 (9%)
IFN-beta	22 (52%)
GA	2 (5%)
Immunosuppressive drugs	14 (34%)

EDSS, Expanded Disability Status Scale;

DMT, disease modifying therapy

^aAt study entry

*****p*<0.0001

Table 2. Primers and probe used in the JC real-time PCR assay.

Oligonucleotide sequences (5'- 3')	Target
TAQJ1 5' AGT GTC CAT TTG AGG CAG CAA 3'	T antigen
TAQJ2 5' GGC ACA TGG CAA TGC TGT T 3'	T antigen
TAQJ3 5' ACA GTG TCC ATT TGA GGC AGT AAA 3'	T antigen
TAQJ4 5' GGC ACA TGG CAA TGC AGTT 3'	T antigen
JC-P 6-Fam – TGAATCCAGGCCACCCCAGCC - Tamra	T antigen

Table 3. Frequency of side effects.

Very common (> 10%)	n	%	Common (1%-10%)	n	%
Exhaustion	20	47.6	Tachicardia	4	9.5
Urinary/upper respiratory infections	14	33.3	HSV labialis	4	9.5
Headache	15	35.7	Mild urticaria	3	7.1
Fever	8	19	Relapse	3	7.1
Itch	8	19	Oral Afte	2	4.7
Arthralgia	6	14.2	Nausea	2	4.7
Face cutaneous rash	6	14.2	Sweating	2	4.7
			Alopecia	2	4.7
			Chills	1	3.3
			Herpes Zoster	1	3.3
			Pneumonia	1	3.3
			Epileptic seizure	1	3.3
			Serum Liver Enzyme Elevation	0	0

Table 4. MS patients who had detectable JC viral loads during 18 months of treatment with natalizumab. The patients who got JCV positive in plasma at month 6 and 13, reversed to negativity at month 7 and 14, respectively. The only patient who resulted consecutively JCV positive in plasma at month 12 and 13, discontinued natalizumab.

Time of testing	Urine no. positive/no. tested	Plasma no. positive/no. tested
Baseline	11/42 (26%)	0/42 (0%)
At month 1	12/42 (29%)	0/42 (0%)
At month 6	12/42 (29%)	1/42 (2%)
At month 12	13/42 (31%)	1/42 (2%)
At month 13	13/42 (31%)	2/42 (5%)
At month 18	3/19 (16%)	0/19 (0%)

Table 5. Absolute number (a.n.) and percentage (%) of circulating immune cell subsets during one-year natalizumab treatment: mean and SD are reported, with the significant comparison of the month 1, 6 and 12 with the baseline. The values recorded at months 2-5 and 7-11 are omitted for clarity. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, non significant.

	Baseline	At month 1	At month 6	At month 12
Leukocytes	5675±1854	6874±1660***	7494±1577	7327±1112
Eosinophils a.n.	98±72	218±113**	263±146	323±218
Eosinophils %	1.8±1.2	3.3±1.8*	3.6±1.9	4.5±2.9
Lymphocytes a.n.	1562±531	2645±784****	2967±763	3015±645
Lymphocytes %	28.3±7.5	39.7±8.3****	39.7±6.8	41.3±7.8
B cells a.n.	199±118	557±248****	636±215	643±231
B cells %	12.2±5.8	20.0±7.0***	21.5±5.4	20.5±5.3
NK cells a.n.	168±70	288±120***	309±132	378±189
NK cells %	11.4±6.0	11.0±4.9	11.0±5.2	12.1±5.4
T cells a.n.	1192±442	1805±654****	1963±551	2057±435
T cells %	75.5±6.5	67.7±6.1***	66.7±5.7	66.6±6.1
CD4+ T cells a.n.	709±269	1059±371****	1151±338	1168±330
CD4+ T cells %	59.1±12.2	57.4±11.3 ns	56.5±15.5	56.7±11.7
CD8+ T cells a.n.	463±255	736±390**	735±308	844±390
CD8+ T cells %	36.7±10.4	38.8±9.7 ns	37.3±8.5	39.0±9.3
NK-like T cells a.n.	59±64	82±84 ns	74±62	87±80
NK-like T cells %	4.6±4.6	4.5±5.3 ns	3.7±3.0	4.0±3.7

Figure 1

