



# Early reduction of the splicing factor2/alternative splicing factor: a cellular inhibitor of the JC polyomavirus in natalizumab-treated MS patients long before developing progressive multifocal leukoencephalopathy

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## Abstract

Natalizumab is effective against relapsing-remitting multiple sclerosis (MS) but increases the risk of progressive multifocal leukoencephalopathy (PML), which is caused by the activation of the JCV polyomavirus. SF2/ASF (splicing factor2/alternative splicing factor) is a potent cellular inhibitor of JCV replication and large T-antigen (T-Ag) expression. We reported that SF2/ASF levels in blood cells increase during the first year of natalizumab therapy and decrease thereafter, inversely related to T-Ag expression, and suggested a correlation with JCV reactivation. Here, we report SF2/ASF levels of longitudinal blood samples of two patients undergoing natalizumab therapy, who developed PML while monitored, in comparison to natalizumab-treated controls and to one-off PML samples. After 6 months of therapy, SF2/ASF levels of the two cases were reduced, instead of increased, and their overall SF2/ASF levels were lower than those from natalizumab controls. Since SF2/ASF inhibits JCV, its early reduction might have a role in subsequent PML. We are aware of the limitations of the study, but the uniqueness of serial blood samples collected before and after PML onset in natalizumab-treated patients must be stressed. If confirmed in other patients, SF2/ASF evaluation could be a new and *early* biomarker of natalizumab-associated PML risk, allowing an 18–24-month interval before PML onset (presently ~5 months), in which clinicians could evaluate other risk factors and change therapy.

**Keywords** Natalizumab · Progressive multifocal leukoencephalopathy, PML · Splicing factor2/alternative splicing factor, SF2/ASF · JCV polyomavirus · T-antigen · PML risk · Multiple sclerosis

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## Introduction

Natalizumab is effective against relapsing-remitting multiple sclerosis (MS, Tintore et al. 2019) but increases the risk of progressive multifocal leukoencephalopathy (PML, Mill and Mao-Draayer 2018). Retrospectively, 78% of PML patients have a presymptomatic phase, recognizable at magnetic resonance imaging ~5 months before symptoms (Scarpazza et al. 2019). PML is caused by the JCV polyomavirus (Assetta and Atwood 2017), a ubiquitous opportunistic pathogen, which rarely infects lytically oligodendrocytes, causing PML. Natalizumab mobilizes pre-B and mature B cells from bone marrow to the periphery, possibly spreading JCV

(Chalkias et al. 2014). Natalizumab alters transcriptional profiles of blood cells, especially of genes related to immune response, signaling, adhesion, and T and B cell metabolism (Lindberg et al. 2008). PML risk factors are the presence of anti-JCV antibody, previous immunosuppressant therapy, and natalizumab treatment duration > 24 months (Tintore et al. 2019; Mill and Mao-Draayer 2018).

Since JCV is ubiquitous and PML occurs rarely, multiple barriers must exist against disease development. PML occurs in the context of profoundly impaired cell-mediated immunity; natalizumab, preventing normal lymphocyte trafficking, is one of the most important factors contributing to the breakdown of neuroimmunosurveillance (Berger and Korolnik, 2005). The drug alters the expression of genes related to immune response, signal transduction, adhesion, and metabolism relevant for T and B lymphocytes, thus increasing the odds of JCV infection and/or replication in the brain (Lindberg et al. 2008).

The SF2/ASF (splicing factor 2/alternative splicing factor) is a key cellular regulator of alternative splicing (Zhao et al. 2015), which inhibits JCV replication, blocking the expression of large T-antigen (T-Ag, Uleri et al. 2013). Conversely, T-Ag suppresses SF2/ASF transcription, revealing a molecular SF2/ASF and T-Ag interplay in JCV control, and suggesting another mechanism of JCV reactivation in patients at PML risk (Craigie et al. 2015).

We reported that SF2/ASF levels in blood cells of MS patients vary during natalizumab therapy, increasing during

the first year, and decreasing thereafter, inversely related to T-Ag (Uleri et al. 2017), and suggested its role in JCV reactivation.

Here, we report data from longitudinal observation of two MS patients undergoing natalizumab therapy, who developed PML while monitored.

## Methods

### Cases

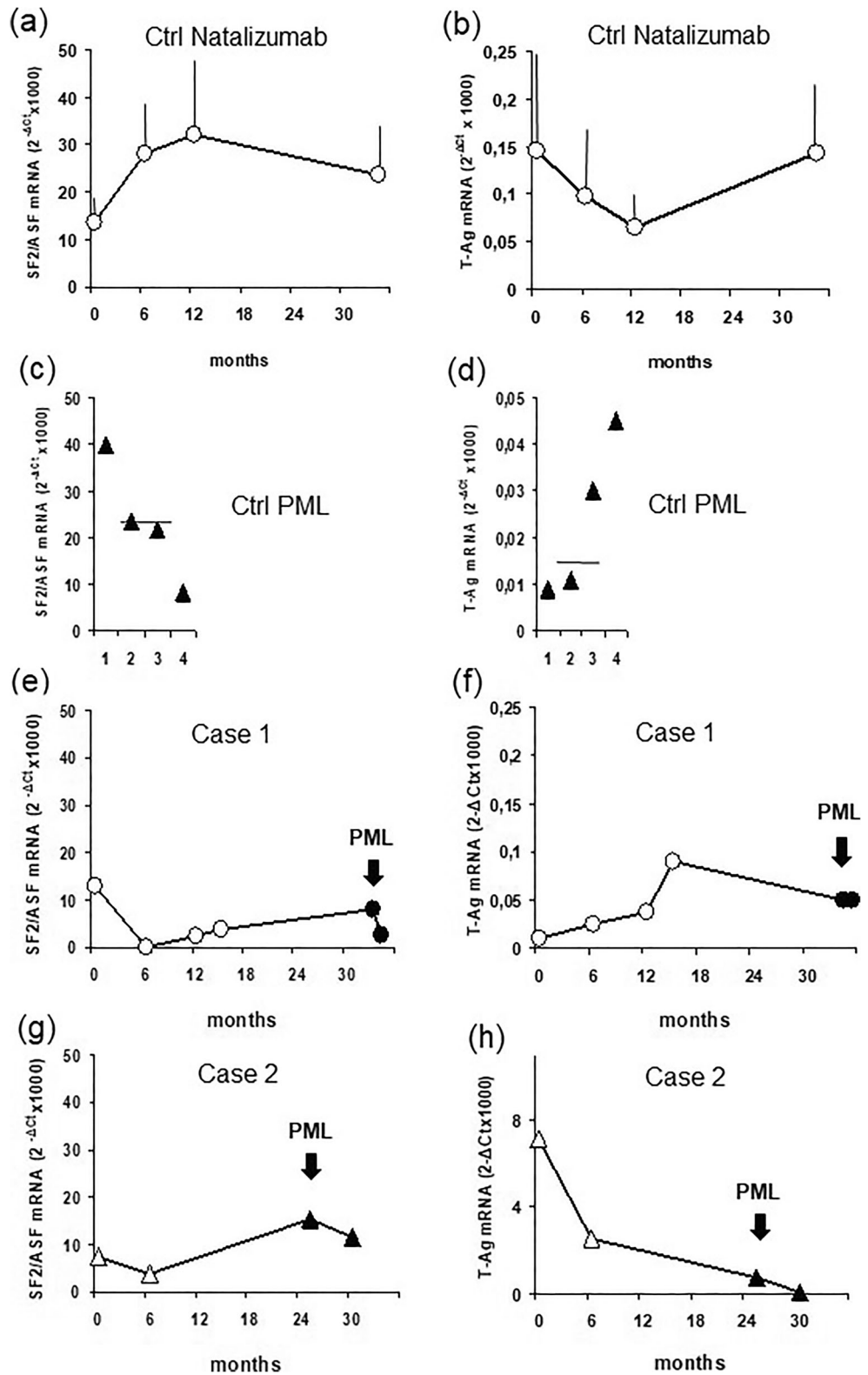
Case 1: female patient, with 22 years of MS at enrolment, and PML onset after 34 months of natalizumab therapy. Case 2: female patient, with 3 years of MS at enrolment, and PML onset after 25 months of therapy. Serial blood samples were collected within a longitudinal study of MS patients undergoing de novo natalizumab therapy. The five MS patients closest for enrolling in the same time interval and location were selected as natalizumab controls. Four one-off samples of PML cases were included as PML controls. Samples were treated as published (Uleri et al. 2017; Gerevini et al. 2019). Demographic and clinical-pathological data of the patients are reported in Table 1. The study has been performed on already stored samples of the two cases, who had been treated years ago, when the knowledge regarding natalizumab-associated risks and the advisability to discontinue the therapy after > 24 months on the

**Table 1** Demographic and anamnestic data of cases and controls

	NZB controls	Case 1	Case 2	PML controls
No of subjects	5	1	1	4
Sex	3 F, 2 M	F	F	4 F
Age at MS onset (mean ± SD)	25.8 ± 6.1	21	24	21.2 ± 9.4
MS years at Time 0 (mean ± SD)	11.2 ± 4.6	22	3	//
MS type	RRMS	RRMS	RRMS	RRMS
MS years at PML onset	//	26	5	16.2 ± 5.2
EDSS at Time 0 (mean ± SD)	4.2 ± 1.1	7	1.5	//
EDSS at PML onset (mean ± SD)	//	7.5	3.5	4.8 ± 1.0
Previous therapy (number of patients)	IFNβ 1a (3) IFNβ 1a/MTX (1) IFNβ 1a/IFNβ 1b/MTX/GA (1)	IFNβ 1a/MTX	IFNβ 1a	IFNβ 1a (1) IFNβ 1b (1) IFNβ 1a/FI (1) IFNβ 1a/FI/GA/DMF/AZB (1)
JCV-DNA copies/ml*	nd	24	73	27 ± 16

NZB, natalizumab; SD, standard deviation; RRMS, relapsing-remitting *multiple sclerosis*; EDSS, expanded disability status scale; IFN, interferon; MTX, mitoxantrone; GA, glatiramer acetate; FI, fingolimod; DMF, dimethyl fumarate; AZB, alemtuzumab; \*in cerebrospinal fluids [all patients were anti-JCV antibody-positive by STRATIFY JCV (Lee et al. 2013)]; nd, not done

**Fig. 1** Levels of SF2/ASF and of T-Ag mRNAs in blood cells from MS patients exposed to natalizumab therapy and from PML cases. **a, c, e, and g** SF2/ASF transcripts; **b, d, f, and h** T-Ag transcripts. **a and b** Natalizumab-treated MS patients; mean values and standard deviations. **c and d** One-off control samples from already established PML cases. **e and f** Case 1, natalizumab-treated patient, who got PML, while monitored. The arrow indicates PML onset, and the black symbols indicate ongoing PML. **g and h** Case 2, natalizumab-treated patient, who got PML, while monitored. The arrow indicates PML onset, and the black symbols indicate ongoing PML. The data are expressed according to the  $2^{-\Delta\Delta Ct}$  method (Uleri et al. 2017). See text for details



drug, if previously treated with immunosuppressive agent, was not yet accessible. The study was approved

by the Brescia Ethical Committee Board (protocols 2713 of 24/05/2017 and NP0 of 07/18/2018).

## mRNA extraction, retro-transcription, and real-time RT-PCR

mRNAs were extracted, retro-transcribed, and amplified with SF2/ASF-specific and T-Ag-specific primers (Uleri et al. 2017). Parallel RNA samples were amplified without retro-transcription, to detect contaminant DNA. For each sample, the Ct values of the gene of interest were compared to those of the glyceraldehyde-3-phosphate dehydrogenase gene. The data were expressed according to the  $2^{-\Delta C_t}$  method (Uleri et al. 2017).

## Results

### Opposite patterns of SF2/ASF and T-Ag expression in natalizumab-treated patients

During therapy, SF2/ASF levels of blood cells of MS patients changed, showing a bell-shaped pattern, increasing during the first year (mean increase of 2.4-fold at 12 months), then declining (Fig. 1a). Conversely, the T-Ag levels showed a U-shaped pattern (Fig. 1b), fully in line with our findings in a Sardinian cohort (Uleri et al. 2017).

The opposite behavior of SF2/ASF and T-Ag levels occurred also in the one-off PML control samples (Fig. 1c, d); their SF2/ASF levels were in the range of natalizumab controls, being T-Ag levels were lower in the PML patients with higher SF2/ASF.

### Early decrease of SF2/ASF levels in the two patients who will develop PML

In both cases, SF2/ASF levels decreased within the first 6 months of therapy, instead of increasing, then partially recovered, until PML onset. Few weeks after PML onset, SF2/ASF levels of both patients decreased again (Fig. 1e, g).

Also in terms of step-wise comparison, the SF2/ASF levels of the cases were lower than those of natalizumab controls, and differences were highly significant (Fig. 2a). Along ~ 3

years of observation, their overall samples had much less SF2/ASF levels than overall samples from natalizumab controls ( $p = 0.0004$ ). Interestingly, after 6 months of therapy, the two cases had only 1/14 of the SF2/ASF levels of natalizumab controls ( $p = 0.028$ ).

The pre-natalizumab values of T-Ag mRNAs were highly different in the two cases, being > 50-fold higher in case 2, who developed PML at 25 months of therapy, than in case 1, who developed PML after 34 months. This suggests that JCV may have been activated already in case 2, before starting natalizumab therapy (Fig. 1f, h).

In line with the opposite behavior of SF2/ASF and T-Ag levels, the two cases had T-Ag levels higher than the natalizumab controls. However, T-Ag values were low, and a statistically significant difference was observed only at 6 months of therapy (Fig. 2b).

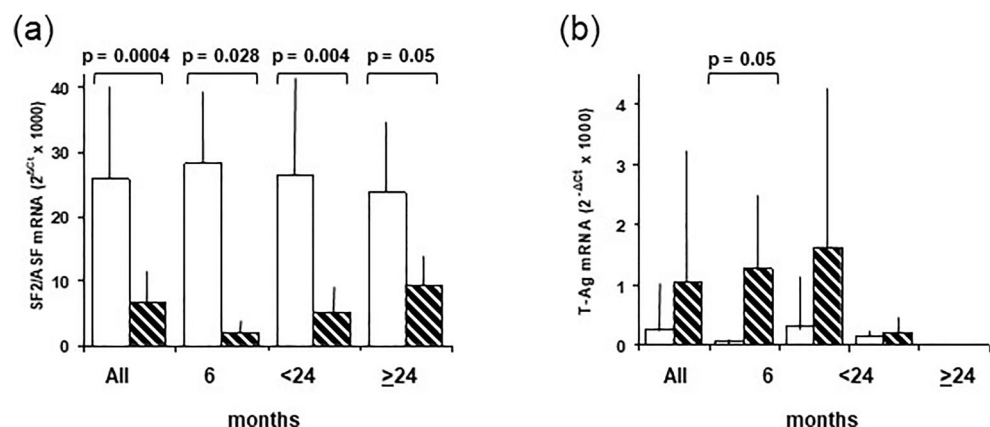
## Discussion

The increased risk of developing PML is a major limitation to natalizumab use (Mill and Mao-Draayer 2018). To reduce PML risk and to decide therapy discontinuation, clinicians evaluate the anti-JCV sero-status, even though viremia and cell-mediated anti-JCV response were detected in seronegative individuals (Mill and Mao-Draayer 2018).

In this case report, we confirm, in another cohort, our previous finding (Uleri et al. 2017) that natalizumab therapy initially stimulates, then inhibits SF2/ASF, potent cellular inhibitor of JCV, with a bell-shaped pattern. Conversely, T-Ag levels show a U-shaped pattern (Fig. 1a, b). Therefore, a consequence of SF2/ASF reduction could be JCV reactivation, thus promoting PML onset.

We had the unique opportunity to analyze two MS patients, who developed PML while enrolled in a longitudinal study. The most apparent finding was a drop in SF2/ASF levels within 6 months from therapy start. Along the ~ 3 years of observation, their overall SF2/ASF levels were much lower than those of natalizumab controls. Accordingly, their T-Ag

**Fig. 2** Step-wise comparison of SF2/ASF and T-Ag levels during time of the two PML cases and the natalizumab-treated control patients. **a** SF2/ASF transcripts; **b** T-Ag transcripts. The statistical significance ( $p$ ) was calculated by the unpaired two-tailed  $t$  test. Open bars, natalizumab-treated control patients; striped bars, PML cases. The data are expressed according to the  $2^{-\Delta C_t}$  method (Uleri et al. 2017). See text for details



levels were higher than the controls. Importantly, in the two cases, SF2/ASF reduction occurred after 6 months of therapy and 19 and 28 months before PML onset, respectively.

We are aware of the limitations of a study of the two cases, but the uniqueness of serial samples collected before and after PML onset in natalizumab-treated patients must be stressed. Since 2009, 56 cases of natalizumab-associated PML occurred in Italy (Scarpazza et al. 2019). To the best of our knowledge, only our two cases received PML diagnosis while enrolled in a longitudinal study. If confirmed in other patients, SF2/ASF evaluation could be a new and *early* biomarker of natalizumab-associated PML risk. It would allow an 18–24-month interval before PML onset (much longer than the present 5-month presymptomatic phase, Scarpazza et al. 2019), in which clinicians could evaluate other risk factors and change therapy.

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**Compliance with ethical standards** The study was approved by the Brescia Ethical Committee Board (protocols 2713 of 05/24/2017 and NP0 of 07/18/2018).

**Conflict of interest** The authors declare that they have no conflict of interest.

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