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Assessment of the risk of polyomavirus JC reactivation in patients with immune-mediated diseases during long-term treatment with infliximab

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Abstract Polyomavirus JC (JCV) reactivation causing progressive multifocal leukoencephalopathy is a main concern during biological therapies. Here, JCV reactivation in patients suffering from immune-mediated diseases after a long-term treatment with anti-tumor necrosis factor alpha (TNF-α) inhibitor infliximab was investigated. Peripheral mononuclear blood cells (PBMC), plasma and urine samples were obtained from 61 immune-mediated diseases patients treated or not with infliximab in combination with steroid and other immunomodulators and from 20 healthy donors. JCV DNA was transiently detected in 12 PBMC of 40 patients at different doses of infliximab with a higher prevalence than that of the 21 patients untreated. Conversely, a stable JCV positivity in urine of treated and untreated patients was detected. Sequencing the noncoding control region (NCCR), all samples exhibited the archetype structure with few mutations in transcriptional factor binding regions. The consequence of anti-TNF-α treatment on viral persistence was examined monitoring Torquetenovirus viremia and investigating the TNF-α-induced microRNA regulators of transcriptional factors, with a binding site on NCCR. Although infliximab treatment in this study did not affect directly JCV reactivation, further investigation on host factor (s) regulated by it will be of warranty in the understanding the mechanism(s) that may affect viral persistence.

Keywords Infliximab · Polyomavirus JC · microRNA · Torquetenovirus · PML

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Introduction

The immune modulatory therapies such as using monoclonal antibodies have revolutionized the treatment of autoimmune inflammatory diseases in recent years (Chan and Carter 2010; Getts et al. 2010). However, the evidence that their use can increase the risk of the reactivation of polyomavirus JC (JCV) and its association with progressive multifocal leukoencephalopathy (PML) is a growing health concern (Carson et al. 2009; Kedar and Berger 2011; Major 2009, 2010). Although

natalizumab and efalizumab, two monoclonal antibodies used in multiple sclerosis and inflammatory bowel disorders and severe plaque psoriasis, respectively, seem to have the highest risk for the development of PML, PML can occur also with the prolonged use of other biologic agents (Major 2010). Recently, this lethal disease of the brain, resulting from lytic infection of the glial cells in severely immunosuppressed patients, has been reported in a patient with rheumatoid arthritis after long-term treatment with infliximab, a chimeric anti-tumor necrosis factor alpha (TNF-α) monoclonal antibody (Kumar et al. 2010). Infliximab has been approved by the US Food and Drug Administration for the treatment of rheumatoid arthritis, psoriasis, Crohn's disease, and other immune-mediated disorders. Although the use of infliximab has increased the quality of life of patients affected by these immune-mediated diseases sometimes its prolonged use has been associated with lymphoma and reactivation of viral and bacterial infections (Bongartz et al. 2006). The effect of TNF-α block produces a decreased expression of some pro-inflammatory cytokines (Maini and Feldmann 2002) modifying the immune surveillance and enhancing the possibility of a virus reactivation (Bellizzi et al. 2010). To date, however, the mechanism that leads to the PML disease remains elusive. Although individual immunomodulatory agents can affect the immune system in different ways, a common prolonged time of treatment seems to be a main patient risk for PML (Major 2010). JCV is a non-enveloped double-stranded covalently closed circular DNA virus widespread in the human population with a genome divided into two coding regions physically separated by the noncoding control region (NCCR; Imperiale and Major 2007). The NCCR is variable and contains promoter/enhancer regions that have been shown to be potentially targetable by several cellular transcriptional factors (Marshall and Major 2010; Raj and Khalili 1995). Some studies indicate that the rearrangements of the JCV NCCR are important to the development of PML, but the initiating events are yet unknown (Ciappi et al. 1999; White and Khalili 2011; Yogo et al. 2008). Our aim was to investigate the presence of JCV in patients with immune-mediated diseases received up to 55 doses of infliximab and to study the possible changes of JCV NCCR in order to monitor the risk of the viral reactivation that can be influenced by its variation during treatment. Also, the possible effect of the anti-TNF-α therapy on features related to the immunity response that could be of interest in the viral reactivation was checked by exploiting the viremia of persistent widespread Torquetenovirus (TTV) residing and multiplying mainly in hematopoietic cells and that, in the past, it has also been suggested its association with autoimmune diseases (Focosi et al. 2009; Maggi and Bendinelli 2009; Gergely et al. 2006; Zhong et al. 2002). Moreover, it was also investigated whether infliximab treatment exerted some effect on the level of microRNAs (miRNAs) expression. This was considered of interest because miRNAs are short, non-coding RNAs that control post-transcriptionally the protein synthesis interfering with mRNA and play a critical role in the innate and adaptive immunity, as well as in cell proliferation and inflammation (Bartel 2009; Bi et al. 2009).

Materials and methods

Patients In this study, 61 patients with immune-mediated diseases (rheumatoid arthritis, systemic vasculitis, seronegative spondyloarthropathies) enrolled at the Immunoallergology Unit, Policlinico di Careggi of the University of Florence were chosen (Table 1). Forty patients (group 1) were regularly treated with 3–5 mg/kg of infliximab every 8 weeks and had received 20.6±15.1 number of infliximab infusion upon enrollment. The infliximab infusion doses reported in Table 1 are those that the patients received at the time of blood and urine sample collection. The majority of these patients were also receiving concomitant therapy with a daily dose of prednisone (9.3±2.9 mg) or 6-methylprednisolone (8±2.7 mg) and methotrexate (10.7±0.5 mg). Twenty-one patients (group 2, disease controls) not treated with infliximab but receiving a daily dose of prednisone (10±0.7 mg) or

Table 1 Characteristic of patients with immune-mediated diseases studied

Group	Number of patients	Mean age, years (range)	Gender (M/F)	Disease[a]	Number of infliximab infusion at enrollment	Timing of infusion
1	15	37.7 (25–80)	8/7	Yes	1–10	Every 8 weeks
	9	31 (21–64)	7/2	Yes	11–20	Every 8 weeks
	5	47.2 (42–51)	3/2	Yes	21–30	Every 8 weeks
	5	43.8 (27–60)	3/2	Yes	31–40	Every 8 weeks
	6	45.3 (35–69)	4/2	Yes	>41	Every 8 weeks
2	21	46 (29–72)	9/12	Yes	–	–
3	20	46.2 (17–72)	7/13	–	–	–

M male, *F* female

[a] The immune-mediated diseases of the examined patients included rheumatoid arthritis, systemic vasculitis, seronegative spondylarthropathies

6-methylprednisolone (10.9±1.3 mg) were also enrolled. The control group (group 3) included 20 healthy blood donors (Table 1). Informed consent was obtained from all subjects enrolled in the study.

JCV PCR quantification JCV infection was assessed by extracting DNA from 2.0×10^6 peripheral blood mononuclear cells (PBMC), 200 μl of plasma and urine with the High Pure PCR Template Preparation Kit (Roche) and amplifying 100 ng of DNA in triplicate with a real-time PCR assay on the T antigen (forward 5′ GAG CAG CTT AGT GAT TTT CTT AGG 3′ and reverse 5′ CAC CAA AAC AAA AGA ACA CAG GTG 3′ JCV-specific primers) of the viral genome using SYBR green chemistry. Each reaction was carried out with negative controls (no template) and DNA standards (diluted to contain 10^1–10^6 copies per milliliter) of a plasmid containing the amplicon cloned. The sensitivity of the assay was 100 copies per milliliter of plasma and urine or per microgram of PBMC DNA.

NCCR sequencing A nested PCR was used to obtain the NCCR product to be sequenced. Briefly, 100 ng of total DNA was amplified employing two pairs of primers: first pair of primers, forward 5′ CCC TAT TCA GCA CTT TGT TCC 3′ and reverse 5′ CCC GTC TAC ACT GTC TTC ACC 3′, generating a 530-bp DNA fragment; the second pair of primers, forward 5′ GCC TCC ACG CCC TTA CTA CTT C 3′ and reverse 5′ CGT GAC AGC TGG CGAAGA 3′, that amplified a portion of the first amplicon generating a fragment of 328 bp. The PCR products were purified using the PCR Purification Kit (Qiagen) and were sequenced using the BigDye Terminator Cycle-Sequencing Ready Reaction (Applied Biosystems, CA). The sequences were analyzed and edited using BioEdit 5.0.9 (Tom Hall of Ibis Therapeutics, Carlsbad, CA).

TTV PCR quantification TTV infection was assayed by DNA extracted from 200 μl of plasma samples and testing the viral loads by using a single step universal TaqMan real-time PCR assay as previously described (Maggi et al. 2005). The sensitivity of the assay was 100 copies per milliliter.

MiRNA RT-PCR quantification Total RNA was isolated from 2.0×10^6 PBMC by using the mirVana miRNA Isolation Kit (Ambion), and the miRNA expression was measured and quantified with the specific miRNA TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) according to the manufacturer‘s protocol and normalized by U6 miRNA levels.

Statistical analyses Data were analyzed by using the χ^2 test. Associations between variables were determined by applying Spearman’s correlation coefficient. The *P* value was calculated to test the significance of correlation, and a value less than 0.05 was considered to be statistically significant.

Results

Clinical characteristics of the 61 patients and 20 healthy donors involved in this study are reported in Table 1. Age and sex was equally distributed in the three groups of subjects. Group 1 (infliximab-treated patients) was divided into five subgroups accordingly to the number of infusions received (Table 1). All treated patients did not experience any adverse events and resulted responders to infliximab therapy. No significant differences in rates of improvement for the different infliximab indications were observed.

JCV DNA in blood and urine of study patients All the patients were assessed for JCV infection using a quantitative real-time PCR assay as described in “Materials and methods.” As reported in Table 2, 12 out of 40 patients (30%) of group 1 were JCV positive in PBMC showing a higher prevalence than that of the patients not treated with infliximab (group 2, 9%) and that of the healthy subjects (group 3, 5%). Of note, a statistically significant difference was observed only between infliximab-treated patients and healthy donors (χ^2, $p<0.05$). Moreover, just five out of 40 patients of group 1 and only one patient of group 3 were positive for JCV DNA in plasma samples (Table 2). Among the patients of group 1, four out of five were JCV DNA positive in both PBMC and plasma separated from the same blood sample. The viral load in the PBMC and plasma of the different groups was low and did not significantly differ. All groups exhibited an high prevalence of JCV in urine samples (72%, 28%, 55% for group 1, 2, and 3, respectively) with a significant difference between group 1 and 2 (χ^2, $p<0.01$). Furthermore, the range of viral load in the urine was higher than that in PBMC and plasma (Table 2). Collectively, time and doses of infliximab treatment did not correlate with significant variations in JCV detection in the study patients. When the positive samples were repeated for the JCV assay in PBMC, plasma, and urine, a constantly positive detection was shown only in urine at subsequent time point (data not shown) consistent with a JCV replication either undetectable or occurred episodically at a low level.

NCCR sequences To investigate the possible mutations that can occur during the treatment, the JCV NCCR of all positive samples was sequenced. All the JCV NCCR sequences were compared to structural organization of the non-pathogenic archetype belonging to type 1, which was the genotype of JCV present in all samples as judged also by the sequencing the VP1 region segment (Agostini et al. 1996, 2001). Results showed that all the JCV NCCR samples had an archetype structure (Fig. 1). However, mutations in cellular transcriptional factors binding sites as well as few irrelevant mutations were identified (data not shown) in positive samples of group 1. These mutations involved the Spi-B (G30A mutation), SP-1 (A166G/T mutation), and

Table 2 JCV quantitative detection on PBMC, plasma and urine of study patients

Group	JCV[a]					
	PBMC		Plasma		Urine	
	Positive/ examined (%)	Copies/μg DNA	Positive/ examined (%)	Copies/ml	Positive/ examined (%)	Copies/ml
1	6/15	3.3×10^3 (8×10^2–9.3×10^3)	2/15	1.4×10^3 (2×10^2–2.7×10^3)	12/15	1.4×10^7 (5×10^4–9.3×10^7)
	2/9	9.6×10^3 (2.3×10^2–1.7×10^4)	1/9	2.2×10^2 (5.7–7.7×10^2)	8/9	1.2×10^7 (1.7×10^5–4.7×10^7)
	1/5	3.6×10^3	0/5	Nd	2/5	4.1×10^6 (1×10^3–2×10^7)
	2/5	9.5×10^3 (1–1.8×10^3)	1/5	2×10^2	4/5	3.9×10^7 (5.7×10^5–8.4×10^7)
	1/6	1.1×10^3	1/6	2×10^2	3/6	1.1×10^7 (3.5×10^5–9×10^7)
	Total, 12/40 (30%)		Total, 5/40 (12%)		Total, 29/40 (72%)	
2	2/21 (9%)	1.7×10^3 (7×10^2–2.8×10^3)	0/21 (0%)	Nd	6/21 (28%)	6.7×10^6 (1×10^3–4×10^7)
3	1/20 (5%)	9×10^2	1/20 (5%)	1.9×10^2	11/20 (55%)	2.9×10^5 (1×10^3–2.3×10^{76})

[a] Viral loads are expressed as mean of copy numbers obtained in the positive samples of each group. In parentheses are the range of copy numbers
Nd not determined

NF-1 (C211G/T mutation) binding sites. In particular, among the 12 NCCR sequences obtained from PBMC, two had all these three changes and three showed only one (NF-1 change). The NF-1 change was also present in one out of five NCCR sequences obtained from the plasma. Finally, among 29 NCCR sequences obtained from urine, one showed all

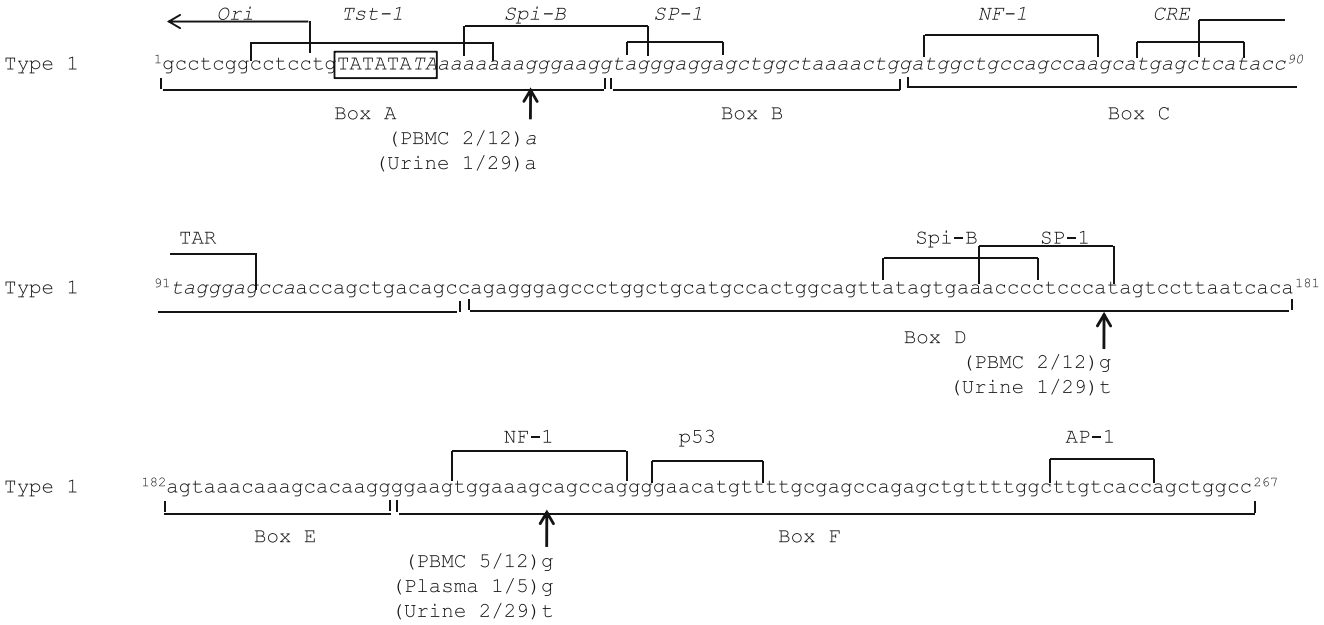

Fig. 1 NCCR structure mutations found in this study. Box *A–F* are shown below the sequence (Imperiale and Major 2007). The sequences analyzed were obtained from 12 PBMC, five plasma, and 29 urine of the JCV DNA positive patients of group 1. The mutations are indicated by the *arrow*. The number of samples with the specific position mutated/total number of samples analyzed for PBMC, plasma, and urine is shown. Only two out of 12 PBMC and one out of 29 urine samples exhibited all three mutations at the same time. Transcriptional factor binding sites are indicated above the sequence. *Ori* origin of DNA replication. TATA sequence is marked with a *box*

three of these changes and one only the NF-1 changed. Of note, the PBMC and urine samples, in which the same mutations were identified, were obtained from the same patients. Although the mutations were located mainly in the binding sites of the transcription factors reported as the lymphoid-specific regulator of the JCV gene expression (Marshall et al. 2010; Monaco et al. 2001), none of these have been previously found in the Mad-1 strain or in other PML-associated strain present in the virus database.

Evaluation of the effect of anti-TNF-α on viral persistence Next, it was of interest to investigate whether the anti-TNF-α effect could actually create a favorable environment for persistent viruses modulating their viral load in blood. As reported in Fig. 2a, the rate of TTV infection was collectively variable among the patients treated or not with infliximab and the healthy controls, with a range of prevalence of 82–86%. Analyzing the TTV viremia in the patients of group 1 is noteworthy; a positive correlation between viral load and the number of infusion performed (Spearman's Rho of 0.299, $p < 0.05$) was observed.

Effect of anti-TNF-α on miRNA expression Based on these results and from the evidence that blocking TNF-α can affect gene expression related to immune response, it was chosen to assess the expression levels of cellular regulatory miRNA. To this end, the expression level of the outmost miRNAs involved in the immune response and deregulated in rheumatic patients, miRNA-146a, -150, -155, and -223, were quantified. The fold changes of the miRNA expression level observed in the infliximab-treated and -untreated immune-mediated disease patients compared to that observed in the healthy control group were reported in Fig. 2b. These levels were lower in the patients treated with infliximab than that observed in patients untreated, with a significant reduced value for miRNA-146a and -155. Furthermore, in the patients treated with infliximab, the miRNA-146a expression level was constantly detected under that of the healthy controls. Conversely, miRNA-150, -155, and -223 levels remained up to 2.5 log higher than that of the healthy controls.

Discussion

Although these results derive from relatively small groups of patients, the infliximab long-term treatment, monitored in this study, did not seem to exert foremost effects on the possible reactivation of JCV associated to the development of PML. However, it was observed that the viral DNA in PBMC was higher in infliximab-treated patients compared to the untreated patients and healthy controls. Moreover, few nucleotide mutations, located in cellular transcriptional factors binding sites of NCCR region, and never found in JCV associated to PML, were observed in the patients of the first group.

So far, the presence of JCV DNA in the peripheral blood cells of healthy and diseased individuals has been well

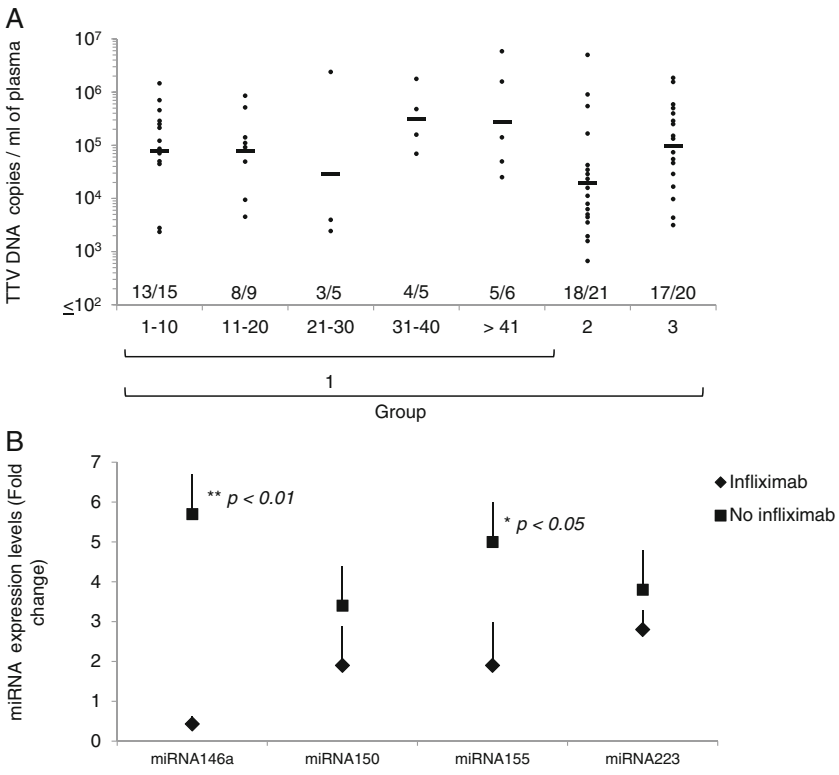

Fig. 2 TTV load and microRNA expression level in immune-mediated disease patients in the presence or absence of infliximab treatment. **a** Individual TTV load in plasma of the patients of each group is reported. *Horizontal bar* represents the mean of viral load. The number of TTV positive patients/total number of patients analyzed is shown. **b** miRNA expression level is expressed as fold change between miRNA expression level obtained in immune-mediated disease patients with respect to that obtained in healthy donors. Values shown are the means ± standard deviation of three independent experiments. *Asterisks* indicate that the miRNA-146a and -155 expression level observed in infliximab-treated patients was significantly different from that of untreated patients (Student's *t* test) at $^{**}p<0.01$ and $^{*}p<0.05$, respectively

documented leading to the hypothesis of their responsible role for viral dissemination to the central nervous system during persistence (Dorries et al. 2003; Houff and Berger 2008; Monaco et al. 1996). Recently, nucleotide changes in Spi-B and NF-1 binding sites of the NCCR of JCV after infliximab treatment in pediatric patients affected by Crohn's disease were reported (Bellizzi et al. 2011). The authors concluded that these changes could be a virulence marker of JCV reactivation during monoclonal antibodies treatment. Conversely, another study provided reassurance on the safety of short-term infliximab treatment in adult Crohn's disease patients with respect to JCV reactivation (Lavagna et al. 2007). In our study, although a role for the concomitant treatment with steroids and other immunomodulators could not be excluded, it seemed that cell-associated JCV occurred transiently more frequently during the anti-TNF-α treatment. Consistent with these results, in a previous study, a JCV cell-associated subclinical reactivation was reported in patients treated with natalizumab (Chen et al. 2009). The mechanism mediated by infliximab and involved in viral reactivation is still unknown. In this regard, one possible explanation could be ascribed to the increase of the hematopoietic cells carrying JCV in a latent state in circulation migrating from the bone marrow by the downregulation of the adhesion molecules expressed on their surface as a consequence of the TNF-α blocking mechanism (Maini and Feldmann 2002). Thus, even if infliximab acts in a different way in respect to that described for other monoclonal antibodies such natalizumab, its final effect on the hematopoietic cells may be analogous (Major 2009). However, the possibility that changes in the levels of cytokines such as interleukin-1 (IL-1), IL-6, and interferon-γ (IFN-γ) mediated by the anti-TNF-α activity (Bellizzi et al. 2010), inducing an immunosuppression, may have facilitated viral dissemination in the blood, is not to be excluded also. The possible effect of the infliximab treatment on the antiviral behavior generated by the changes of pro-inflammatory cytokines was investigated by monitoring the TTV load. The use of this virus was considered of interest because TTV is present in plasma of nearly all the people, producing no obvious diseases, and for the evidence that changes in its viral load, most likely generated and maintained by the hematopoietic cells in the blood, may offer a good approximation of immune functional status (Maggi and Bendinelli 2009; Maggi et al. 2010; Focosi et al. 2010). The variation in TTV load observed after different doses of the infliximab treatment confirmed that a reduction in the antiviral control, likely related to the decreased IFN-γ levels (Bellizzi et al. 2010), could have occurred but also provided additional evidences on the possible role of hematopoietic cells in the JCV replication. On the other hand, the TNF-α block inducing changes in the level of pro-inflammatory cytokines could have modulated also the activities of transcription factors such as NF-κB, C/EBPβ, and AP-1 which have been described as potential factors involved in the regulation of JCV gene expressions (Bartel 2009; Marshall and Major 2010; Wollebo et al. 2011). In this context, of note, it has been hypothesized that the upregulation of these cellular transcription factors, observed also during natalizumab treatment, could be associated to JCV replication (Lindberg et al. 2008). Investigating the miRNAs, as potential regulatory factors of gene expression, overall the upregulated miRNA levels observed in the patients affected by immune-mediated diseases with respect to that of the healthy controls confirmed what was described in other studies in patients affected by rheumatoid arthritis and other autoimmune diseases (Furer et al. 2010). However, in the patients treated with infliximab, the miRNAs levels were constantly lower than that obtained in patients not treated with infliximab, with the level of miRNA-146a under that of healthy control. Noteworthy miRNA-146a has been described as one of the negative regulators of the induction of NF-κB activity, a critical mediator of the immune response and also a well-known activator of JCV replication (Ma et al. 2011). Collectively, these results confirm that the anti-TNF-α activity exerted by infliximab may deregulate specific miRNA expressions as reported in other studies (Furer et al. 2010; Pedersen et al. 2009). Thus, the investigation of interaction between miRNAs and transcriptional factors might be of interest in order to clarify the possible implication of the TNF-α-induced miRNAs as modulators in JCV reactivation.

In light of the described models of JCV latency in hematopoietic cells of the bone marrow as source that allows JCV to circulate, and the potential role of TNF-α in the JCV reactivation (Wollebo et al. 2011), further studies should be performed in order to understand the real risk of the infliximab treatment and to identify the possible factor(s) that induce(s) the viral changes involved in the PML development.

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

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