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ORIGINAL ARTICLE

TSPO PET imaging of natalizumab-associated progressive multifocal leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML) is a severe infection of the CNS caused by the polyomavirus JC that can occur in multiple sclerosis patients treated with natalizumab. Clinical management of patients with natalizumab-associated PML is challenging not least because current imaging tools for the early detection, longitudinal monitoring and differential diagnosis of PML lesions are limited.

Here we evaluate whether translocator protein (TSPO) PET imaging can be applied to monitor the inflammatory activity of PML lesions over time and differentiate them from multiple sclerosis lesions. For this monocentre pilot study we followed eight patients with natalizumab-associated PML with PET imaging using the TSPO radioligand ^{18}F-GE-180 combined with frequent 3 T MRI. In addition we compared TSPO PET signals in PML lesions with the signal pattern of multiple sclerosis lesions from 17 independent multiple sclerosis patients. We evaluated the standardized uptake value ratio as well as the morphometry of the TSPO uptake for putative PML and multiple sclerosis lesions areas compared to a radiologically unaffected pseudo-reference region in the cerebrum. Furthermore, TSPO expression *in situ* was immunohistochemically verified by determining the density and cellular identity of TSPO-expressing cells in brain sections from four patients with early natalizumab-associated PML as well as five patients with other forms of PML and six patients with inflammatory demyelinating CNS lesions (clinically isolated syndrome/multiple sclerosis).

Histological analysis revealed a reticular accumulation of TSPO expressing phagocytes in PML lesions, while such phagocytes showed a more homogeneous distribution in putative multiple sclerosis lesions. TSPO PET imaging showed an enhanced tracer uptake in natalizumab-associated PML lesions that was present from the early to the chronic stages (up to 52 months after PML diagnosis). While gadolinium enhancement on MRI rapidly declined to baseline levels, TSPO tracer uptake followed a slow one phase decay curve. A TSPO-based 3D diagnostic matrix taking into account the uptake levels as well as the shape and texture of the TSPO signal differentiated >96% of PML and multiple sclerosis lesions. Indeed, treatment with rituximab after natalizumab-associated PML in three patients did not affect tracer uptake in the assigned PML lesions but reverted tracer uptake to baseline in the assigned active multiple sclerosis lesions.

Taken together our study suggests that TSPO PET imaging can reveal CNS inflammation in natalizumab-associated PML. TSPO PET may facilitate longitudinal monitoring of disease activity and help to distinguish recurrent multiple sclerosis activity from PML progression.

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Abbreviations: DWI = diffusion weighted imaging; IRIS = immune reconstitution inflammatory syndrome; JCV = polyomavirus JC; PML = progressive multifocal leukoencephalopathy; SUVR = standardized uptake value ratio

Introduction

In recent years an increasing number of progressive multifocal leukoencephalopathy (PML) cases—a severe opportunistic infection of the CNS caused by polyomavirus JC (JCV)—have been observed as a complication of multiple sclerosis therapy with natalizumab.[1] While natalizumab-associated PML *per se* is a rare complication with an estimated incidence of ~3.99/1000 patients as of March 2020,[2] natalizumab is widely used in multiple sclerosis therapy and as a result, more than 832 cases have now been confirmed across the globe.[2] Clinical management of patients with natalizumab-associated PML has remained challenging with a fatality rate of the disease of >20% and varying levels of disability in those who survive.[2,3] Management of surviving patients is further complicated by immune reconstitution inflammatory syndrome (IRIS) and the reoccurrence of multiple sclerosis-related disease activity at variable time points after cessation of natalizumab therapy.[4–6] Tools that allow specific monitoring of PML activity are thus of critical importance for clinical management.

MRI can reveal tissue alterations caused by PML and is the current gold standard for brain imaging of PML patients.[7] While MRI has proven to be a valuable tool for clinical diagnosis and management of these patients, important aspects such as the early detection and the long-term monitoring of PML activity as well as the unequivocal differentiation of multiple sclerosis and PML-related inflammatory activity have remained challenging.[8] One approach to meet these challenges can be PET imaging of radioligands that bind to the mitochondrial 18 kDa translocator protein (TSPO).[9] TSPO PET is based on the observation that mononuclear phagocytes upregulate the expression of the TSPO protein upon activation[10,11] and has been used to measure CNS inflammation in multiple sclerosis[12–15] and other neurological conditions.[16,17] This is relevant in the context of natalizumab-associated PML as previous histological analyses showed that mononuclear phagocytes (either locally activated microglial cells or invading monocyte-derived macrophages) are important contributors to the inflammatory reaction in this disease.[18,19] Here we use the TSPO radioligand ^{18}F-GE-180 that has been shown to detect CNS inflammation in multiple sclerosis and glioma patients and is also susceptible to changes in blood–brain barrier permeability.[20–22] For this purpose we first verified that TSPO-expressing mononuclear phagocytes accumulate in brain lesions of patients with natalizumab-associated PML and subsequently explored whether TSPO PET can be used for longitudinal monitoring of inflammatory activity in natalizumab-associated PML and differentiation of PML progression from re-occurrence of multiple sclerosis disease activity.

Materials and methods

Tissue specimens and histopathological analysis

For histopathological analysis brain specimens obtained from autopsies and biopsies of patients with natalizumab-associated PML (n = 4, Table 1), other forms of PML (n = 5) or clinically isolated syndrome/multiple sclerosis (n = 6) were included. Non-multiple

sclerosis PML patients (mean age 31.7 ± 2.9 years, information not available for two samples) included two cases of HIV/AIDS-associated PML. The clinically isolated syndrome/multiple sclerosis patients consisted of two males and four females (mean age 38.2 ± 15.8 years) and showed a disease duration smaller than 6 months for five of the cases. Brain sections from autopsies ($n = 4$) of patients who died from non-neurological diseases served as controls (two females and two males, mean age 40.8 ± 7 years). Their use for scientific purposes was in accordance with institutional ethical guidelines and was approved by the ethics committee of the University of Göttingen (Germany) and the University of Geneva (Switzerland).

Immunostaining for TSPO (PBR), mononuclear phagocytes (CD68) and activated astrocytes (GFAP) was carried out on 3-µm thin sections of paraffin-embedded tissue blocks using the following primary and secondary antibodies: monoclonal rabbit anti PBR (1:100, Abcam, ab109497), monoclonal mouse anti-CD68 (1:100, Dako, M0876) and a chicken polyclonal antibody to GFAP (1:100, Abcam, ab4674). For quantitative analysis, three regions of interest from each area (PML lesion, PML adjacent white matter, PML adjacent grey matter, actively demyelinating multiple sclerosis lesion) as well as from white matter regions in non-inflammatory control samples were chosen using Pannoramic viewer software (3D-Histech) and quantified using the ImageJ 'Cell Counter' plugin.

Study cohort

In this pilot study we prospectively included multiple sclerosis patients with a diagnosis of natalizumab-associated PML. The local Ethics Committee (IRB no. 48-15) and the German Radiation Protection Committee authorized the study (BfS no. Z 5-22463/2-2015-006). Written informed consent was given by all patients before participation in the study. The following inclusion criteria were applied: age between 18 and 85 years, a diagnosis of multiple sclerosis based on the 2010 revision to the McDonald criteria, and a diagnosis of PML based on the 2013 PML diagnostic criteria.[23] All patients presented with clinical worsening and tested positive for JCV DNA in the CSF. Exclusion criteria were: major psychiatric and/or other medical disease, pregnancy, unrelated exposure to >15 mSv per year, and general contraindications to PET/MRI. Eight patients with natalizumab-associated PML and relapsing remitting multiple sclerosis were included (for clinical characteristics see Table 2 and Supplementary Fig. 1). The TSPO binding affinity status was analysed based on polymorphism genotyping at the Department of Psychiatry of the University Hospital Regensburg. For this purpose, genomic DNA was extracted from 4 ml of whole blood with QIAamp DNA blood maxi kit (Qiagen) according to the manufacturer's protocol. DNA quality was assessed utilizing optical absorbance and gel electrophoresis. In line with previous studies using the ^{18}F-GE-180 tracer[20,24] we could detect increased tracer uptake in patients with different TSPO binding affinity statuses. Our study included one low-affinity binder [Patient 1, mean PML lesional standardized uptake value ratio (SUVR) 1.60], and one medium-affinity binder (Patient 7, mean PML lesional SUVR 1.65 ± 0.20), while all remaining patients were high-affinity binders (mean PML lesional SUVR 2.11 ± 0.58).

MRI

MRI was acquired on a Magnetom Skyra 3T scanner (Siemens Healthineers) with a slice thickness of 3 mm at the Institute of Clinical Radiology, LMU Munich using protocol defined sequences (axial T_2-weighted and T_2-FLAIR, T_1-weighted including contrast enhanced T_1-weighted images). The mean interval between the PET and the correlated MRI scan was 2.9 ± 3.3 days. Serial MRI were complemented by external MRI scans, including standard sequences [T_1-weighted, T_2-weighted, T_2-FLAIR and diffusion-weighted imaging (DWI)]. PML lesions and multiple sclerosis lesions of all available MRIs (analysing all sequences, including DWI, FLAIR and contrast enhanced MRI) were evaluated by two experienced neuroradiologists (A.G. and M.P.) and classified as PML or multiple sclerosis lesions using standard diagnostic criteria.[25,26] Information on the MRI presentation of multiple sclerosis lesions (number of lesions, lesion location and number of lesions with contrast enhancement) at the time point of PET imaging in PML patients is given in Supplementary Table 1. PML lesions from the serial MRI evaluations were assigned to the following stages taking into account previously suggested MRI criteria[1]: early symptomatic PML before IRIS (increasing lesion size, little or no gadolinium enhancement), IRIS phase (gadolinium enhancement common, punctate pattern and T_1 bright cortical line), post-IRIS phase of PML (no gadolinium enhancement, no MRI features of IRIS, but close temporal proximity, ongoing inflammatory activity and tissue repair), or post-PML (stable phase of persistent tissue defect with atrophy in PML region), as shown in Supplementary Fig. 1.

MRI contrast analysis

For the quantification of contrast enhancement in MRI within the PET lesion area both the non-contrast enhanced T_1-weighted image and the PET-based volumes of interest were co-registered to each patient's contrast-enhanced T_1-weighted image. The non-contrast enhanced T_1-weighted images were automatically segmented with FreeSurfer (v6.0; http://surfer.nmr.mgh.harvard.edu/; accessed 9 August 2021) in order to create patient-specific, binary masks of the whole-brain white matter and the lateral ventricles. For Patient 4, manual segmentation was applied. To enable quantitative comparisons of signal intensities between the contrast enhanced-T_1-weighted and non-contrast enhanced images, the signal of each voxel was normalized to the mean signal intensity within the ventricle mask and a contrast ratio score was computed. We defined a ratio score of >1.2 to be contrast positive, with this threshold yielding the strongest signal-to-noise separation. Representative images with overlayed co-registered masks

Table 1 Demographic and clinical characteristics of natalizumab-associated PML cases included in the histopathological analysis

Patient no.	Sample	Age/ Sex	NTZ infusions (n)	Last NTZ infusion to biopsy/autopsy (weeks)	First PML symptoms to biopsy/autopsy (months)	Clinical stage	PLEX/IA	JCV in CSF (copies/ml)
1	Autopsy	49/F	17	9	2	MS-PML	PLEX (2×), IA (3×)	Positive
2	Autopsy	60/M	40	14	2	MS-PML	PLEX (2×), IA (3×)	450 000
3	Autopsy	40/M	58	13	3	MS-PML-IRIS	PLEX (5×)	112
4	Biopsy	25/F	27	14	3	MS-PML-IRIS	IA (5×)	Positive

F = female; IA = immunoadsorption; M = male; MS = multiple sclerosis; NTZ = natalizumab; PLEX = plasma exchange.

Table 2 Demographic and clinical characteristics of PML cases included in the PET imaging analysis

Patient no.	Age/ Sex	EDSS	NTZ infusions (n)	Last NTZ infusion to Dx of PML (w)	PET1 to Dx of PML (m)	PET2 to Dx of PML (m)	PET3 to Dx of PML (m)	PLEX/IA at Dx of PML	JCV in CSF at Dx of PML (copies/ml)	JCV-Ab index value in serum before Dx of PML	Clinical IRIS to Dx of PML (w)	Dx of PML to Rituximab (m)
1	40/M	4.0	71	15	0	–	–	–	2500	3.06	0	–
2	55/F	3.5	58	8	2	–	–	PLEX (2×)	309	2.36	6	–
3	51/M	4.5	79	3	3	9	–	–	44	1.08	17	–
4	56/M	3	48	0	5	12	–	IA (5×)	26	3.14	6	–
5	35/F	4	50	3	11	19	26	PLEX (7×)	30	3.55	5	12
6	40/F	4	46	0	12	20	44	PLEX (5×)	1440	3.70	7	14
7	37/M	4	52	2	19	52	–	IA (5×)	377	Positive	6	20
8	50/F	6	66	6	25	35	–	PLEX (5×)	1300	0.81	5	45

Dx = diagnosis; EDSS = Extended Disability Status Scale; F = female; IA = immunoadsorption; M = male; m = months; NTZ = natalizumab; PLEX = plasma exchange; w = weeks. JCV-Ab index value data from Unilabs.

(PET-based volume of interest, above threshold volume) of two patients with contrast enhancing PML lesions are shown in Supplementary Fig. 3.

^{18}F-GE-180 synthesis

^{18}F-GE-180 was produced on a FASTlab™ synthesizer with single-use disposable cassettes provided by GE Healthcare. The obtained radiochemical purity was ⩾95%. Full GMP requirements for tracer synthesis were met. Detailed specifications were published previously.[27]

PET acquisition

A Biograph 64 PET/CT scanner (Siemens Healthineers) at the Department of Nuclear Medicine, LMU Munich was used to scan patients. A low-dose CT scan was acquired for attenuation correction. After intravenous bolus injection of 189 ± 12 MBq ^{18}F-GE-180, emission scans were recorded over 90 min in list-mode. Subsequently, an OSEM2D algorithm was applied for image reconstruction (eight iterations, four subsets, 4 mm Gauss) with a matrix size of 256 × 256 × 109 and a voxel size of 1.336 × 1.336 × 2.027 mm^3. The trans-axial resolution [full-width at half-maximum (FWHM) at 10 cm radial distance and with HI-REZ Option] was 4.8 mm and axial resolution (FWHM at 10 cm radial distance and with HI-REZ Option) was 5.4 mm. For scatter, decay, attenuation, and random counts standard corrections were applied.

PET image evaluation

The PMOD Neuro tool was used for evaluation of the PET data based on 60–90 min summation images after correction of subject motion and co-registration of PET images on T_1-weighted as well as T_2-FLAIR MRI. For anatomical volume of interest definition in PET space, the default processing workflow within PMOD Neuro tool was applied, utilizing T_1-weighted MRI data and the N30R83. Afterwards, each T_1-weighted MRI sequence was normalized to MNI space and subsequently a maximum probability atlas[28,29] was applied. Volumes of interest were then transformed into PET space. An established threshold-based method for the segmentation of focal multiple sclerosis lesions[20,30] was adapted for the delineation of both PML lesions and multiple sclerosis lesions in this study. Therefore, a pseudo-reference region (PRR) within the contralateral cortex was chosen as background volume for segmentation. The same pseudo-reference region was used for PML and multiple sclerosis lesions within each patient. A total of 89 multiple sclerosis lesions from 17 (non-PML) multiple sclerosis patients used to train the diagnostic matrix were taken from Unterrainer *et al.*[20] and delineated as described. The mean SUV of the PRR was similar for both studies [mean SUV ± standard deviation (SD) of the PRR in this study: 0.38 ± 0.03 versus mean SUV ± SD of the PRR in Unterrainer *et al.*[20]: 0.36 ± 0.03]. To understand the temporal reliability of lesion analysis in sequential scans we further analysed the SUV of the PRR in individual patients over time and observed minimal variation of tracer uptake values in the PRR in consecutive scans (Supplementary Fig. 2).

For quantitative analysis of tracer uptake, the maximum uptake was extracted from a hot spot composed of three voxels. The relation of the gadolinium status and corresponding TSPO tracer uptake (mean SUVR ± SD) in individual multiple sclerosis and PML lesions is shown in Supplementary Table 2.

Quantitative analysis of TSPO uptake pattern

To improve inter- and intra-patient comparability of quantitative values, a normalization to standardized uptake values (SUV =

activity concentration × patient weight / injected activity) and SUV ratios (SUVR = SUV / SUVreference) was performed. From SUVR images, first order statistics, texture features, and shape parameters were extracted using an in-house developed software written within the ROOT data analysis framework (version 6.09/01, Cern, Switzerland) integrating algorithms provided within the ITK segmentation and registration toolkit (version 4.11, National Library of Medicine). The following texture features were derived from a grey level co-occurrence matrix: energy, entropy, correlation, inverse difference moment, inertia, cluster shade, cluster prominence, and Haralick's correlation. The included shape parameters were: elongation, roundness, equal sphere radius, flatness, mesh volume and mesh area. For texture analysis, intensity values from SUVR images were discretized using a fixed bin size of 0.05 based on previous studies.[31,32] The cut-off criterion, SUVRMax, was calculated in R [Scatter3D plugin and rgl library (3D Visualization Using OpenGL)] based on the concentration ellipsoid (α) with the level parameter (expected proportion of bivariate-normal observations) set to 0.95. For matrix analysis, lesions were classified as multiple sclerosis lesions, if they localized outside the PML ellipsoid (green). Respectively, lesions were defined as PML lesions if they localized outside the multiple sclerosis ellipsoid.

Statistical analysis

Statistical analysis was done with Prism (Versions 6.0 and 7.0, Graphpad) using one-way ANOVA test (as normal distribution could be assumed), corrected for multiple comparisons using the Turkey or Dunnett's test. Paired t-test (assuming normal distribution, based on D'Agostino-Pearson test) was applied for statistical analysis of multiple sclerosis and PML lesion before and after anti-CD20 therapy.

Data availability

The data that support the findings of this study are available from the corresponding authors (T.K., M.K.) upon reasonable request.

Results

Histopathological analysis of TSPO expression in acute natalizumab-associated PML

To assess whether TSPO imaging could be a suitable approach to measure inflammatory activity in natalizumab-associated PML, we first characterized the spatial and cellular profile of TSPO immunoreactivity in brain sections from four acute natalizumab-associated PML cases as well as five cases with other forms of PML (non-multiple sclerosis PML), six cases of clinically isolated syndrome/multiple sclerosis and four age-matched control cases without an inflammatory CNS disease (Table 1). We observed a marked increase in TSPO immunoreactivity in PML cases that appeared most prominent in the subcortical white matter lesion area and followed a reticular pattern on the macroscale (Fig. 1A) consistent with the multifocal appearance of PML lesions,[23] while TSPO immunoreactivity in active multiple sclerosis lesions appeared more homogeneous and was often most pronounced at the lesion border (Fig. 1B), as previously reported.[11] In natalizumab-associated PML cases the density of TSPO-positive cells was highest within white matter lesions, while the adjacent grey and white matter showed only a moderate increase in relation to control sections (Fig. 1C and D). To determine the cellular identity of the TSPO-positive cells in PML and multiple sclerosis lesions we counterstained the sections with markers for activated mononuclear phagocytes (CD68) and reactive astrocytes (GFAP)—cell populations that have been previously shown to express TSPO upon activation.[10,33] Our analysis showed that the (vast) majority of TSPO-positive cells in PML and multiple sclerosis white matter lesions were activated mononuclear phagocytes. While reactive astrocytes were thus only a minor contributor to TSPO expression in the white matter lesions of natalizumab-associated PML, they constituted a sizeable proportion of the TSPO-positive cells in the adjacent white and grey matter. In all investigated areas, only few TSPO-positive cells expressed neither CD68 nor GFAP, indicating that mononuclear phagocytes and astrocytes are the major TSPO-expressing cell types in natalizumab-associated PML (Fig. 1E and F). Taken together, our histological analysis indicates a close spatial correlation of TSPO expression with CNS inflammation in natalizumab-associated PML with the highest density of TSPO-expressing cells in the highly inflammatory white matter lesions. Activated mononuclear phagocytes, either derived from local microglial cells or from infiltrating blood-borne monocytes, are the major cellular source of TSPO expression in these lesions. These results thus provide a rationale for using TSPO PET imaging to track CNS inflammation in patients with natalizumab-associated PML.

Longitudinal monitoring of inflammatory activity in natalizumab-associated PML using ^{18}F- GE180 TSPO PET

To assess whether increased TSPO expression in natalizumab-associated PML can be leveraged for non-invasive monitoring of CNS inflammation, we performed TSPO PET imaging in eight multiple sclerosis patients (between 35 and 56 years of age) who developed PML during immunosuppressive therapy with natalizumab (after 46–79 infusions) (Table 2). PET imaging ($n = 16$ PET scans in eight patients) was performed using the ^{18}F- GE-180 tracer as previously described[20] in parallel with standardized 3 T MRI scans and accompanied by frequent additional MRI documentation (Supplementary Fig. 1). Using this approach, we assessed TSPO tracer uptake in PML lesions at different time points after PML diagnosis and before, during and after clinical and radiological appearance of IRIS. We found that TSPO PET imaging was able to detect PML-associated inflammation at all stages of the disease and even before contrast enhancement indicative of IRIS appeared on MRI scans. This was apparent in Patient 3, who was treated with natalizumab for highly active multiple sclerosis and was referred to our hospital with progressive dysarthria and paresis of the left arm 3 months after the initial diagnosis of PML based on a cortical hyperintense T_2-FLAIR lesion in the right precentral gyrus on MRI and the detection of JCV DNA in the CSF. TSPO PET imaging at the time of admission to our hospital (+3) revealed a large area with strongly enhanced tracer uptake in the right fronto-parietal lobe. This area of enhanced tracer uptake clearly exceeded the corresponding hyperintense signal on T_2-FLAIR and isotropic images (DWI) on the corresponding MRI (+3), particularly towards the parietal region and contrast enhancement on MRI became only visible 3 weeks later at Month 3.5 (Fig. 2). Likewise on the contralateral (left) side, increased tracer uptake in the absence of altered diffusion restriction could be observed in an area (Fig. 2B) that only became clearly visible as a T_2-FLAIR hyperintense lesion in the follow-up MRI (+3.5). TSPO tracer uptake was still increased at the last documented time point in the post-IRIS phase indicating that despite cessation of lesion increase on MRI and cessation of gadolinium enhancement, an increased inflammatory activity persisted in the lesion area.

To assess whether TSPO PET imaging can also help monitor inflammatory activity in natalizumab-associated PML over time, we measured the mean TSPO SUVR in PML lesions derived from a total of 16 TSPO PET measurements obtained between 0 and 52 months after PML diagnosis. While the extent of the increased tracer

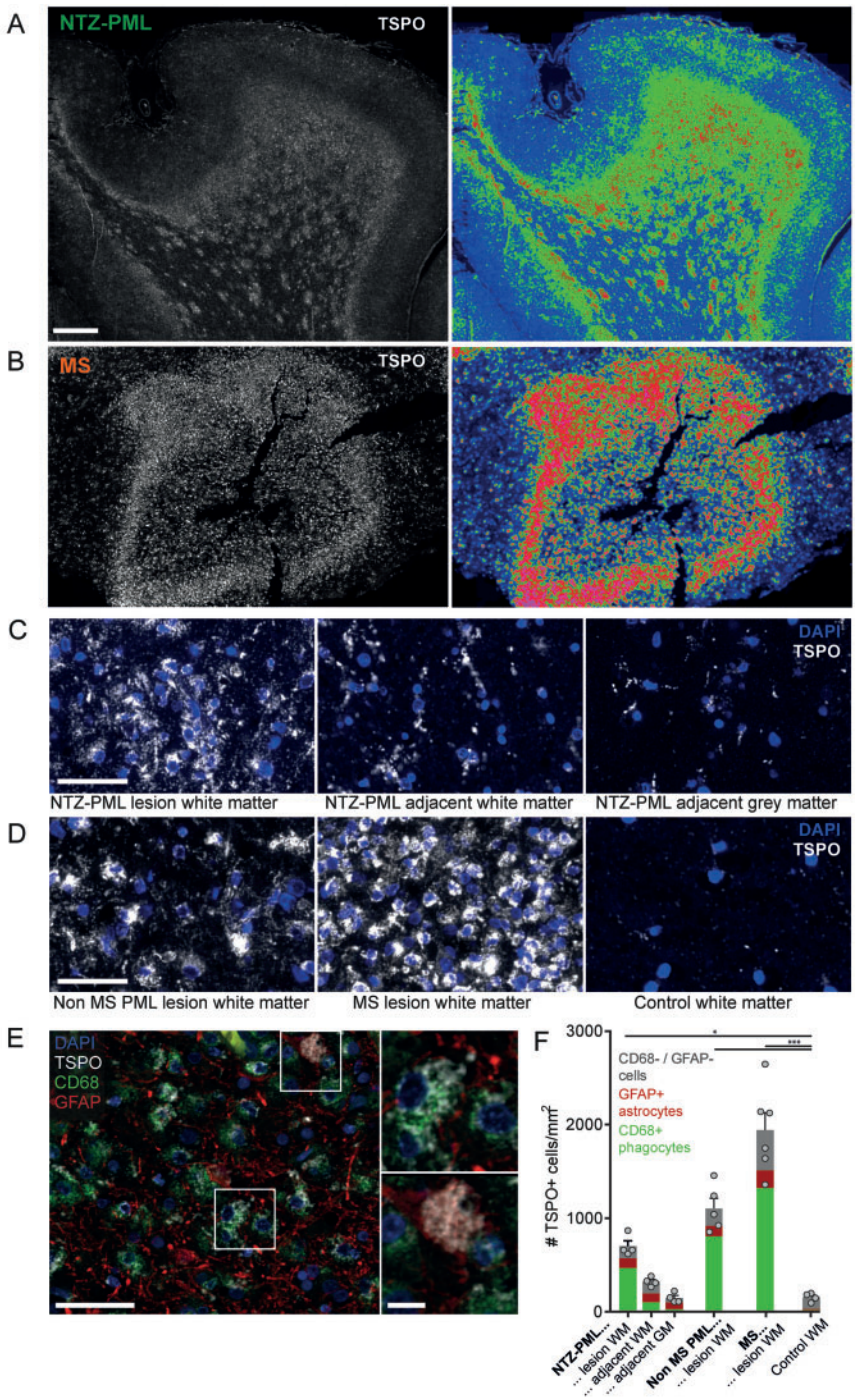

Figure 1 Activated mononuclear phagocytes show prominent TSPO expression in natalizumab-associated PML. (**A**) Immunostaining of brain autopsy tissue from a patient with confirmed natalizumab-associated PML (NTZ-PML) showing increased TSPO expression (TSPO: white) in the subcortical PML lesion area (*left*); heat map representation of the TSPO immunosignal intensity reveals a reticular pattern of TSPO expression in PML lesions (*right*). (**B**) Immunostaining of brain biopsy tissue from a patient with a putative multiple sclerosis lesion (MS) showing strong TSPO expression (TSPO: white) in the lesional white matter (*left*); heat map representation of spherical lesion (*right*). (**C**) Confocal images showing the different densities of TSPO-positive cells (TSPO: white; DAPI: blue) in NTZ-PML lesion white matter (*left*), lesion adjacent white matter (*middle*) and adjacent grey matter (*right*). (**D**) TSPO-positive cells (TSPO: white; DAPI: blue) in non-natalizumab associated PML (non-multiple sclerosis PML) lesion white matter (*left*), putative multiple sclerosis lesion white matter (multiple sclerosis, *middle*) and non-inflamed control white matter (*right*). (**E**) Cell type-specific co-labelling indicates that TSPO is primarily expressed in CD68-positive phagocytes and GFAP-positive astrocytes in NTZ-PML (TSPO: white; DAPI: blue; GFAP: red; CD68: green). *Top right*: High magnification image of a TSPO-positive phagocyte. *Bottom right*: High magnification image of TSPO-positive astrocyte. (**F**) Quantitative analysis of the density of TSPO-positive cells in natalizumab-associated PML (NTZ-PML, $n = 4$ patients), non-natalizumab associated PML (non-multiple sclerosis PML, $n = 5$ patients), putative multiple sclerosis lesions (multiple sclerosis, $n = 6$ patients with clinically isolated syndrome/multiple sclerosis) and control white matter ($n = 4$ cases). Three regions from each area were analysed, respectively. Fraction of the cellular identity of TSPO-positive cells, colour-coded green (CD68-positive phagocyte), red (GFAP-positive astrocyte) and grey (CD68- and GFAP-negative cells). $P < 0.05$ for NTZ-PML lesion white matter, $P < 0.0001$ for non-multiple sclerosis PML and multiple sclerosis as compared to control white matter using one-way ANOVA and *post hoc* testing (Dunnett's multiple comparisons corrected). Only comparisons to control white matter are highlighted; when compared to multiple sclerosis lesion white matter, all groups show a significant difference ($P < 0.0001$). Scale bars in **A** = 2000 μm; **B** = 200 μm; **C** and **D** = 50μm; **E** = 50 μm (*left*) and 10 μm (*right*).

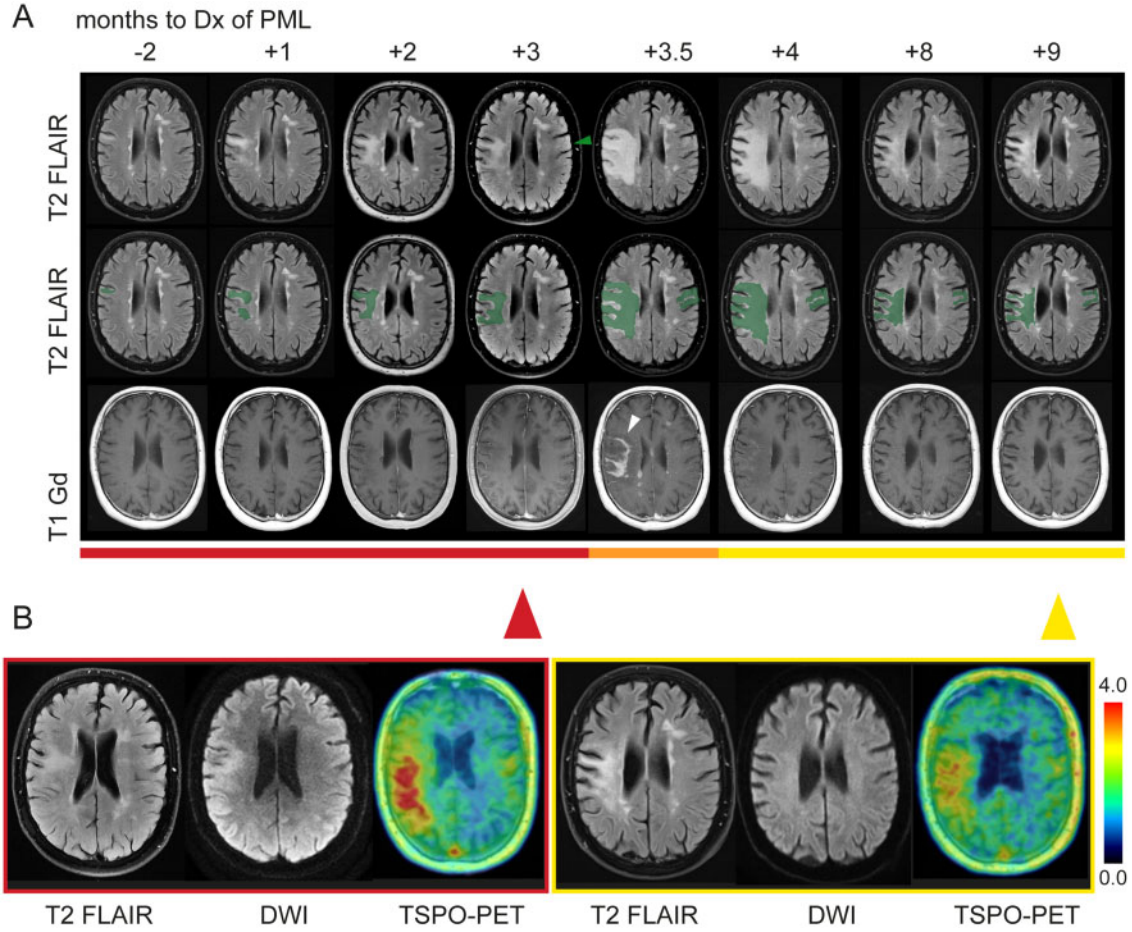

Figure 2 Temporal evolution of MRI and TSPO PET presentation in Patient 3. (**A**) *Top row*: T_2-FLAIR. *Centre row*: T_2-FLAIR (PML lesion is coloured in green to visualize lesion evolution). *Bottom row*: T_1-weighted image after gadolinium. Colour bars indicate the phase of PML (red: PML before IRIS, orange: PML-IRIS, yellow: post-IRIS PML). The PML lesion shows an increase in size up to the IRIS phase when prominent contrast enhancement is seen (white arrowhead). First signal abnormalities compatible with an emerging cortical PML lesion are seen at +3 (green arrowhead). A decrease in lesion size is observed after cessation of IRIS (Months 8 and 9). (**B**) Corresponding TSPO PET, FLAIR and DWI at time points +3 and +9 (red and yellow arrow heads). At +3: DWI hyperintensity marks acute PML lesion. Enhanced tracer uptake markedly exceeds the area of T_2-FLAIR and DWI hyperintensity of the PML lesion. Contralaterally a cortical/subcortical patch of enhanced tracer uptake is also seen in the area of the putative new PML lesion. At +9 DWI isointensity and FLAIR regression demonstrate the typical phase of defect healing. At this point the TSPO PET lesion also shows decrease in size and tracer uptake intensity, that is still elevated compared to normal tissue. *Bottom right*: Colour reference bar indicating SUVR (0.0–4.0) in PET images.

uptake in early PML appeared somewhat variable, our results showed that the slowly progressive decline of TSPO tracer uptake following IRIS remarkably closely followed a standard one-phase decay curve and remained elevated for several years after PML diagnosis (Fig. 3A and C). This is in marked contrast to the temporal evolution of the contrast enhancement measured by MRI in the same lesions that was only detectable during the comparably short IRIS phase of the disease process (Fig. 3A, B and Supplementary Fig. 3). These findings thus indicate that TSPO PET imaging might be suitable for long term monitoring of PML-associated inflammatory activity that extends substantially beyond the PML-IRIS phase. Moreover, the remarkable consistency of the individual measurements with an interpolated decay curve suggests that the inflammatory activity in PML lesions declines in a rather stereotypic and thus predictable manner over time.

A TSPO-based diagnostic matrix differentiates natalizumab-associated PML and multiple sclerosis lesions

To explore whether TSPO PET could reveal distinctive features of CNS inflammation, we selected differentiation parameters for each dimension of lesion features (uptake level, texture, shape) via univariate analysis for their power to separate multiple sclerosis lesions ($n = 89$ lesions from an independent cohort of 17 multiple sclerosis patients taken from Unterrainer *et al.*[20]) from natalizumab-associated PML lesions [$n = 23$ PET lesion analyses (volumes of interest) derived at different time points from 13 PML lesion areas from eight patients]. In this analysis optimal differentiation yield was obtained for a combination of (i) the TSPO SUVRMax, as a measure of the maximal tracer uptake; (ii) the inertia of the lesions texture as a parameter of local signal homogeneity variability; and (iii) equal sphere radius, the shape factor that quantifies the sphericity of the lesion. When we plotted the TSPO-based measurements of these multiple sclerosis and PML lesions, all acquired with the same PET imaging set-up, in a corresponding 3D matrix, distinct lesion clouds emerged (Fig. 4A, B and Supplementary Fig. 4). Using these criteria, >96% of all PML and multiple sclerosis lesion analyses could be differentiated (21 of 23 PML lesion analyses and 87 of 89 multiple sclerosis lesions; Fig. 4B). Moreover, all lesions with a high SUVRMax (>2.9; Fig. 4C) could be clearly separated. The latter is of importance as differentiating highly active multiple sclerosis and PML lesions is critical when facing a patient with newly emerging clinical symptoms after the initial PML episode.

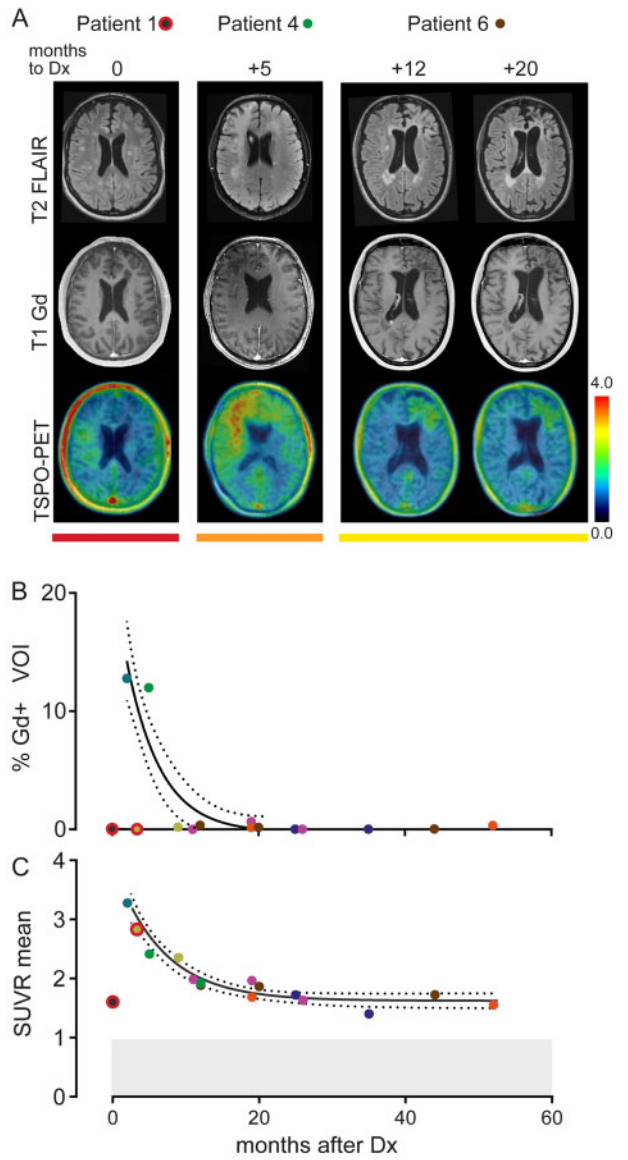

Figure 3 TSPO PET imaging reveals stereotypic decline of TSPO tracer uptake in natalizumab-associated PML. (**A**) Appearance of PML lesions in MRI (*top row*: T_2-FLAIR; *middle row*: T_1-weighted image with gadolinium) and TSPO PET imaging (*bottom row*) at different time points after diagnosis (*top*, months after diagnosis) in patients with natalizumab-associated PML (*left:* Patient 1, *middle:* Patient 4, *right and far right:* Patient 6). Colour reference bar for SUVR (0.0–4.0) in PET images. (**B**) Percentage of gadolinium-positive voxels in T_1-weighted MRI within co-registered PET-derived lesion volumes of individual patients over time showed a short period of contrast enhancement in PML lesions (colour-coded dots represent $n = 8$ patients at PET imaging time points, red circled dots show PML before IRIS, second time point contrast MRI of Patient 4 was not acquired). Interpolated curves, starting at PML-IRIS stage, describe a one-phase decay with 95% confidence intervals. (**C**) TSPO-SUVR of the same PML lesions suggests a stereotypic decline of TSPO tracer uptake over time in natalizumab-related PML (colour-coded dots represent $n = 8$ patients, red circled dots show PML before IRIS). Interpolated curves, starting at PML-IRIS stage, describe a one-phase decay with 95% confidence intervals.

We next assessed whether this TSPO-PET imaging could be helpful in the clinical management of multiple sclerosis patients, who show new clinical symptoms and/or new MRI lesions after diagnosis of a natalizumab-associated PML. While the analysis of the mean TSPO SUVR showed no significant differences between lesions groups (Supplementary Table 2), the analysis of the TSPO-based diagnostic matrix allowed us to assign 12 of 13 lesions that occurred after PML diagnosis in four patients (Patients 5–8) as 'multiple sclerosis lesions'. These included one lesion of a patient (Patient 5) that formed in the brainstem in the immediate vicinity of a pre-existing PML lesion and two new enhancing lesions in Patient 8, with long-lasting JCV DNA positivity in the CSF (Fig. 5A, B and Supplementary Fig. 1). Based on these results and supporting MRI and CSF analysis, we decided to treat three of these patients with an anti-CD20 immunotherapy (rituximab). While TSPO tracer uptake was comparable between PML and the assigned 'multiple sclerosis lesions' prior to treatment, on the follow-up scan between 6 and 32 months after initiation of rituximab therapy, TSPO tracer uptake in all 11 putative 'multiple sclerosis lesions' of treated patients had returned close to baseline. In contrast, uptake in the pre-existing PML lesions remained mostly unchanged, confirming the correct assignment provided by our TSPO-based diagnostic matrix (Fig. 5C). Taken together, our study thus indicates that TSPO imaging reveals characteristic features of CNS inflammation that can be leveraged to identify distinct inflammatory pathologies and differentiate clinical entities.

Discussion

To our knowledge this is the first study assessing TSPO PET imaging as a tool to monitor inflammatory activity in natalizumab-associated PML. We first validated this approach by histological analysis of brain tissue sections that showed a pronounced increase of TSPO-positive cells in white matter lesions of both natalizumab-associated and other forms of PML. The vast majority of the TSPO-positive cells in the lesion area are activated phagocytes, derived either from locally activated microglia cells or from infiltrating blood-borne monocytes. This observation is in accordance with a number of previous studies of TSPO expression in other neuroinflammatory conditions[11,34–36] and supports the notion that TSPO PET can be used to monitor phagocyte activation in PML lesions.

In line with the marked accumulation of TSPO-positive cells in early PML lesions, we observed a robust increase in tracer uptake in early PML lesions. Interestingly, in individual cases, increased tracer uptake was already observed before contrast enhancement became apparent on MRI as part of the IRIS and before PML lesions became clearly visible on T_2-FLAIR or DWI images. These initial observations indicate that TSPO PET might be able to detect PML lesions already during their formation phase—a finding that is consistent with the idea that local microglial cells rapidly sense and react to changes in the brain environment.[37,38] In this context it is interesting to note that TSPO uptake preceding MRI detection of contrast enhancement has also been reported for multiple sclerosis lesions.[39] Such early lesion detection would be of particular importance in a condition like natalizumab-associated PML, in which lesions likely start to form months before clinical symptoms become apparent[1,40] and early diagnosis and cessation of immunosuppression are critical for clinical outcome.[41] Frequent brain MRI scans are the most sensitive and clinically applicable imaging technique for early detection of suspect brain lesions in patients at risk of natalizumab-associated PML. However, a definite diagnosis of such suspect lesions based on MRI alone often remains challenging arguing for multi-modal imaging approaches that include techniques such as TSPO PET that assess defined pathological aspects of such lesions. In this context, it is interesting to note that a recent case report suggested that FDG PET can be used to differentiate hypometabolic PML lesions from hypermetabolic PML-IRIS lesions early in the course of natalizumab-associated PML.[42] Correspondingly ^{1}H magnetic resonance spectroscopy

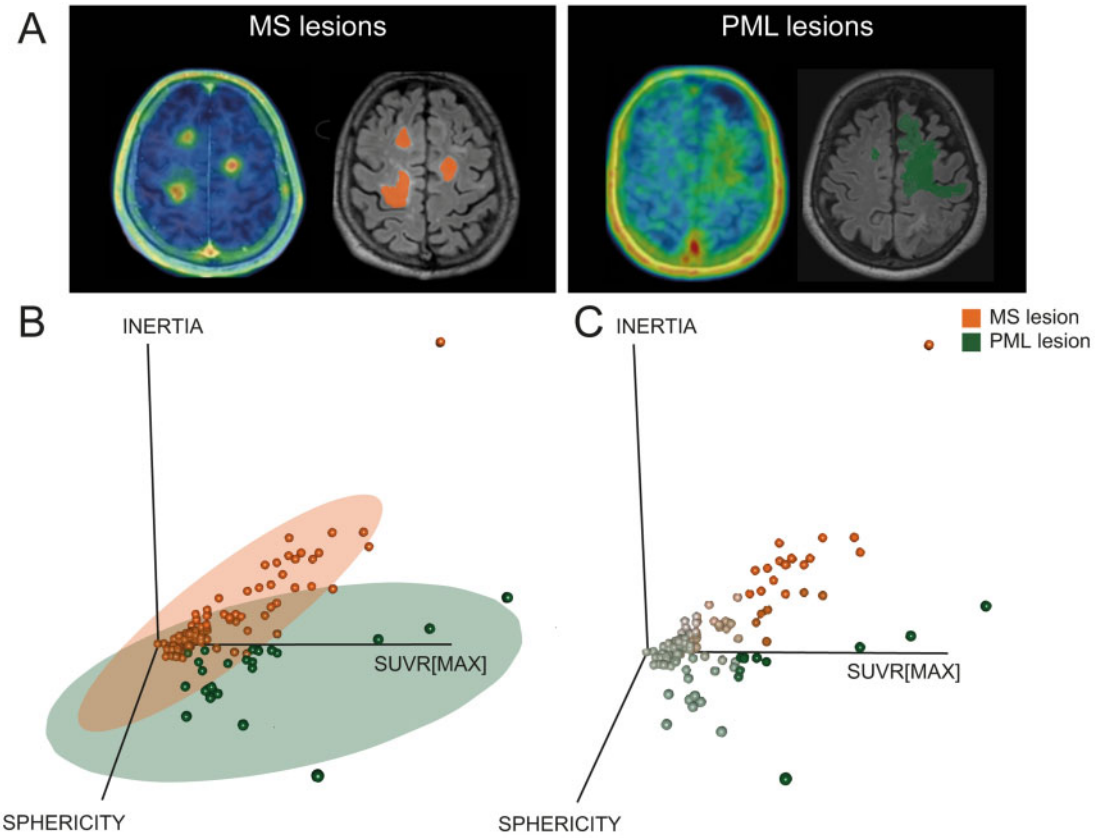

Figure 4 Distinguishing PML and multiple sclerosis lesions using TSPO PET. (**A**) Illustration of multiple sclerosis (MS) versus PML lesion morphology in TSPO PET scans reveals characteristic shape patterns (orange: multiple sclerosis lesion; green: PML lesion). (**B**) Differential clustering of multiple sclerosis versus PML lesions based on 3D diagnostic matrix that combines quantitative imaging parameters of the uptake levels (SUVRMax), as well as the shape (sphericity) and texture (inertia) of the uptake in TSPO PET [orange: $n = 89$ multiple sclerosis lesions from $n = 17$ multiple sclerosis patients previously described by Unterrainer *et al.*,[20] green: $n = 23$ PML lesion analyses (volumes of interest) from $n = 8$ natalizumab-associated PML patients; ellipsoids show 95% confidence region (orange for multiple sclerosis lesions, green for PML lesions)]. (**C**) All lesions with high SUVRMax (>2.9) can be clearly separated (grey: lesions with SUVRMax <2.9, orange: multiple sclerosis lesions with SUVRMax >2.9, green: PML lesion analyses with SUVRMax >2.9).

(^{1}H-MRS) has been shown to detect characteristic metabolic patterns in PML lesions that differentiate disease stages during the course of PML in multiple sclerosis patients.[43,44]

While tracer uptake reaches peak levels during the IRIS phase, we subsequently observed a slow decline of TSPO tracer uptake that, however, remained clearly elevated compared to intact brain tissue for the entire observation period of more than 4 years after PML diagnosis. This temporal profile is in marked contrast to the evolution of gadolinium enhancement that is only present in the IRIS stage and returns to baseline levels within a few weeks thereafter. The presence of TSPO tracer uptake along the entire course of PML raises the possibility that it can be used to stage inflammatory activity of PML lesions and thereby monitor progression of natalizumab-associated PML.[45] In addition, TSPO-PET imaging may thus become useful to monitor treatment responses in natalizumab-associated and other PML cases, for example to new immunotherapies or anti-viral agents.[45–47] Furthermore the remarkably stereotypic decline of tracer uptake argues that in most patients with natalizumab-associated PML, inflammation resolves in a uniform and therefore predictable pattern.

The need to restart multiple sclerosis therapies in patients with natalizumab-associated PML usually emerges when patients start to develop new clinical symptoms months to years after the initial PML symptoms.[6,48] In principle, these symptoms could be caused by a re-emergence of multiple sclerosis activity or a further progression of PML. Differentiating between these two scenarios is obviously critical as they lead to vastly different therapeutic consequences. MRI scans can provide important clues to facilitate this differentiation as a number of MRI features suggestive of PML lesions such as a subcortical location involving U-fibres, ill-defined borders towards white matter but sharp demarcation to the grey matter, T_1-weighted hypointensity, DWI hyperintensity and punctate T_2-weighted hyperintensities with a relative lack of contrast enhancement have been described.[1,49] However, as no single feature is pathognomonic for PML, the differentiation from multiple sclerosis is challenging, in particular when PML lesions are small [25] or when new multiple sclerosis lesions appear in the vicinity of a pre-existing PML lesion (Fig. 5A). As JCV may persist over a long period of time in the CSF or vice versa may be negative in some patients with ongoing PML, repeated CSF analysis alone may not be sufficient to differentiate multiple sclerosis disease activity from PML progression.[1] In these cases our study indicates that TSPO PET imaging can be helpful: first, because measuring tracer uptake in the pre-existing PML lesion allows us to judge its lesional activity (in particular when compared to previous images or the decay curve described here); and second, because the TSPO-based diagnostic matrix we describe here can distinguish the vast majority of PML and multiple sclerosis lesions. We constructed this matrix based on three parameters that quantify distinct traits of maximal tracer uptake [SUVRMax, lesion shape ('sphericity') and the signal texture within the lesion ('inertia')]. The 'sphericity' parameter makes use of the fact that most multiple sclerosis lesions

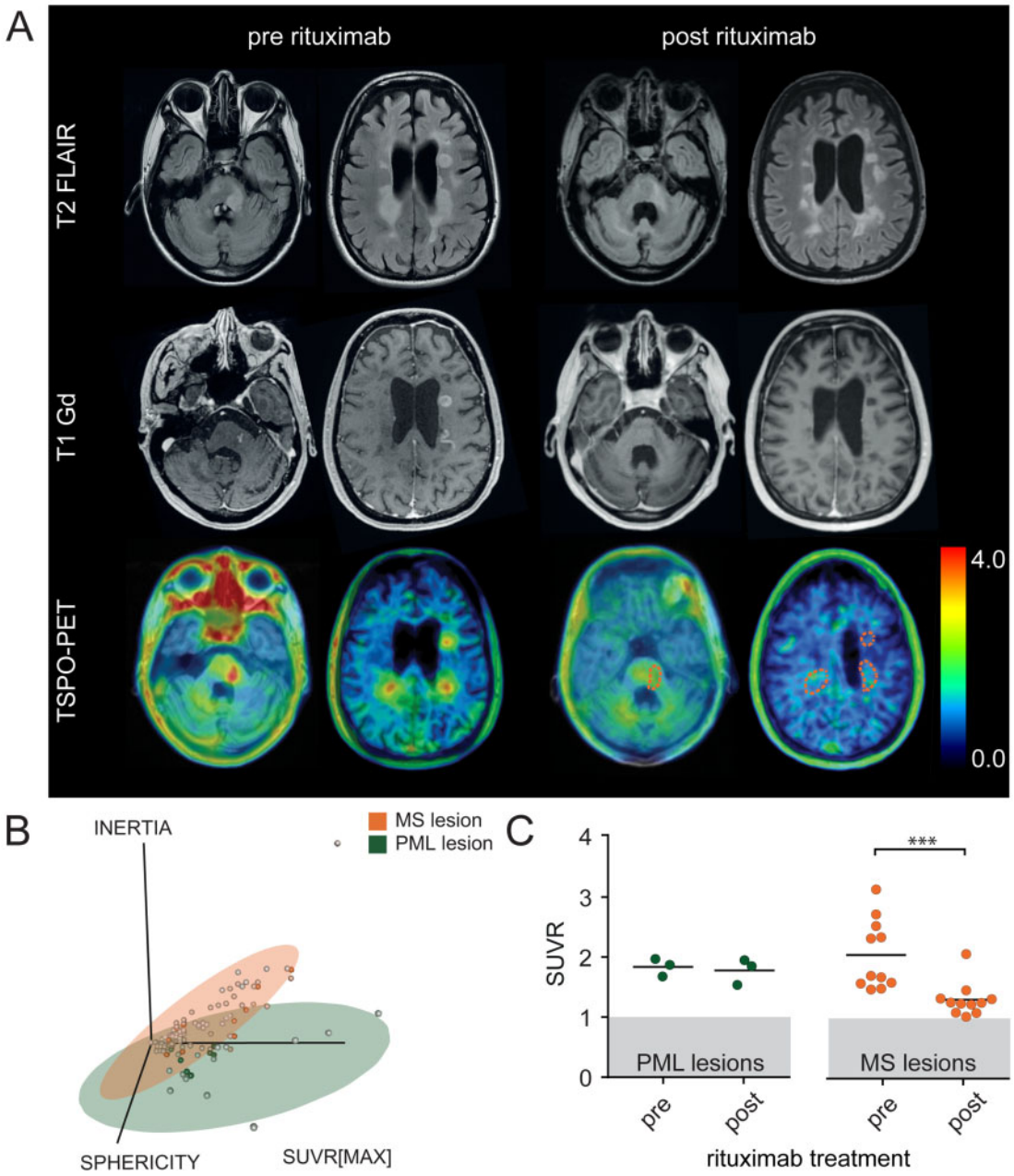

Figure 5 Effect of rituximab treatment in post-IRIS-PML with recurrent multiple sclerosis activity. (**A**) Patient 5 with recurrence of brain lesions in post-IRIS-PML before (*left*) and after rituximab treatment (*right*). *Top row*: T_2-FLAIR shows new lesions in the left cerebral peduncle and in the periventricular white matter. *Middle row*: T_1-weighted images show focal gadolinium uptake in several new lesions. *Bottom row*: Tracer uptake in new lesions (orange circle) and the pre-existing PML lesion area. Reference bar for TSPO-SUVR. (**B**) Quantitative analysis revealed multiple sclerosis-like features of the TSPO tracer uptake in 12 of 13 (92%) new lesions [orange: $n = 13$ lesions from four natalizumab-associated PML patients (Patients 5–8) with new brain lesions after PML] compared to PML lesions [green: $n = 7$ PML lesion analyses from the same scan of the same patients (Patients 5–8); six of seven PML lesion analyses (86%) were identified as PML by matrix analysis]. Previously analysed PML and multiple sclerosis lesions from Fig. 4B are shown in grey for comparison. Ellipsoids show 95% confidence region (orange for multiple sclerosis lesions, green for PML lesions), based on lesions analysed in Fig. 4B. (**C**) Quantitative analysis of TSPO SUVR revealed differential tracer response to rituximab treatment of PML (*left*: mean of lesion analyses in Patients 5–7) compared to assigned multiple sclerosis lesions (*right*: t0: pre-rituximab treatment; t1: post rituximab treatment, Patients 5–7; for multiple sclerosis lesions $P < 0.001$; no significant difference for mean TSPO SUVR for PML lesions versus multiple sclerosis lesions at t0, *t*-test).

appear round or ovoid,[50] while PML lesions often show a more irregular shape.[51] While measuring maximal tracer uptake (SUVRMax) primarily relates the other parameters to the corresponding lesion activity, the 'inertia' parameter leverages the micro-anatomy of PML lesions that compared to most active multiple sclerosis lesions are not confluent sites of increased inflammatory activity but rather clusters of multiple small inflammatory foci that can be readily detected in TSPO imaging (*cf.* Figs 1A, B 3A and Supplementary Fig. 1). While this matrix allowed us to correctly assign most of the newly formed lesions observed in our study patients (Fig. 5), one must keep in mind that such discrimination might be more challenging for very small PML lesions (Supplementary Fig. 4) or unusually large 'tumefactive' multiple sclerosis lesions.

What can we learn from our results regarding the nature of the inflammatory reaction in natalizumab-associated PML? The stereotypic decline of inflammatory activity probably reflects the predictable clearance of the infection by the reconstituted and *a priori* competent immune systems of the affected patients. Yet, while inflammatory activity does decline over time, this process occurs slowly over the course of many months and PML lesions still show elevated tracer uptake at the longest observation points of our study 52 months after PML diagnosis. This is in stark contrast to tracer uptake in multiple sclerosis lesions that approached baseline levels within 6 months after initiation of rituximab therapy (Fig. 5C). This indicates that in PML lesions, major contributors to local innate immune responses such as phagocytes and astrocytes remain in an activated state for years after the initial formation of the PML lesion. This could merely be reflective of an ongoing local adaptive immune response but the emerging roles of innate immune cells in both regulating CNS inflammation and tissue remodelling[52,53] suggests a more active contribution to PML lesion fate that will be important to explore further.

Several limitations have to be considered when assessing our study and its conclusions. First, while a number of different radioligands for TSPO have been generated over recent years, none are without drawbacks.[9,54] In the case of the TSPO ligand ^{18}F-GE180[20,24,30,55,56] that we use here, the most prominent drawback is that it shows a rather low brain penetration across the intact blood–brain barrier in humans.[22] Tracer uptake is therefore likely to be susceptible to alterations affecting blood–brain barrier permeability.[21,57,58] Marked changes of blood–brain barrier permeability, which can be visualized by gadolinium enhancement on MRI, can thus contribute to the enhanced tracer uptake observed during IRIS. However, they are unlikely to explain the substantial tracer uptake preceding contrast enhancement on MRI (Fig. 2) or to underlie the long-lasting tracer accumulation in PML lesions that showed a more prolonged time-course than gadolinium enhancement (*cf.* Fig. 3B and C). In contrast, a contribution of putative 'micro blood–brain barrier damage', which would allow passage of the TSPO tracer but not of gadolinium, is difficult to exclude based on our measurements. One should note, however, that a recent competitive binding study showed a comparably high specific binding of the ^{18}F- GE180 tracer in the brains of multiple sclerosis patients[59] and that our own histological analysis confirms that the spatial distribution of the TSPO target in brain sections from patients with natalizumab-associated PML matches the tracer signal observed in our study. The CNS inflammatory activity we measure here could thus ultimately result from a combination of increased TSPO presence based on the infiltration of monocyte-derived macrophages as well as the activation of local microglial cells and enhanced tracer access based on micro-perturbations of the blood–brain barrier that result from this local inflammation. This might be one explanation why the density of TSPO-positive cells but not the TSPO uptake differ between multiple sclerosis and PML lesions (*cf.* Fig. 1F and Supplementary Table 2). Thus, PET imaging approaches that can more directly assess aspects of blood–brain barrier function might be one approach to better untangle the relative contributions of target expression and target access to the observed tracer signals.[60–62]

A second limitation of our study is that—as PML is a rare complication of natalizumab therapy—it is based on a small sample size of eight patients. Most of these patients were scanned repeatedly so that overall, our analysis is derived from 16 TSPO PET scans. As TSPO PET imaging (not least due to the local tracer synthesis) is a logistically demanding examination technique, these numbers of scans are comparable to other recent (TSPO) PET imaging studies often conducted in more prevalent conditions.[63,64] Still, this is a pilot study performed at a single centre and it will be important to validate the robustness of key parameters that describe the stereotypic decline of inflammatory lesion activity or define the TSPO-based diagnostic matrix in larger patient cohorts and across different PET scanner settings. This is particularly important for the TSPO-based diagnostic matrix that was developed based on a training set of highly active multiple sclerosis lesions,[18] and is thus geared towards the differentiation of gadolinium-enhancing multiple sclerosis lesions from PML lesions.

In conclusion we demonstrate that TSPO PET imaging can provide an approach to monitor inflammatory activity in patients with natalizumab-associated PML. Our histological analysis confirms the rationale for this approach by showing that TSPO-expressing cells, most of them mononuclear phagocytes, accumulate in early PML lesions. TSPO PET imaging in patients shows that inflammatory activity in PML lesions is more extensive than indicated on T_2-FLAIR and DWI and can be monitored throughout the disease course. Enhanced tracer uptake is present from the early stages of disease, peaks around immune reconstitution and subsequently follows a one-phase decay curve. The remarkable adherence of the patients in our study to this decay curve supports the notion that PML activity declines in a rather stereotypic and thus predictable manner after immune-reconstitution in patients with natalizumab-associated PML. Finally, a diagnostic matrix based on the intensity and morphometry of TSPO signals may be used to distinguish PML and multiple sclerosis lesions and help differentiating PML progression from multiple sclerosis re-occurrence.

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Competing interests

A.G. has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Novartis, Biogen, Merck Serono, Sanofi-Genzyme, Roche. T.K. has received speaker and advisory board honoraria from Bayer Healthcare, Teva, Merck, Novartis, Sanofi, Roche and Biogen as well as grant support from Novartis and Chugai Pharma. M.Ke. has been on advisory boards for Biogen, medDay Pharmaceuticals, Novartis and Sanofi, has received grant support from Sanofi and Biogen (including for the initial phase of this study) and speakers fees from Abbvie, Almirall, Biogen, medDay Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi and Teva. The rest of the authors report no disclosures related to this study.

Supplementary material

Supplementary material is available at *Brain* online.

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