Elsevier Editorial System(tm) for The Lancet Neurology

Manuscript Draft

Manuscript Number: THELANCETNEUROLOGY-D-16-00958R2

Title: A decade of lessons learned: PML pathogenesis and risks associated with therapies for MS

Article Type: Review

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Abstract: There are several areas that surfaced from investigations on PML in MS patients that will be explored in this review: 1. The molecular aspects of pathogenesis and cell specific involvement of JCV infection leading to PML, that are generally applicable to all PML cases regardless of various underlying diseases. 2. the central role of magnetic resonance imaging (MRI) in the diagnosis of PML, monitoring treated patients to control its morbidity and advancing our understanding of aspects of its pathophysiology, and 3. new insights of clinical value of early PML detection.

A decade of lessons learned: PML pathogenesis and risks associated with therapies for MS

Introduction

Over one decade has passed since the first report of progressive multifocal leukoencephalopathy (PML) in several multiple sclerosis (MS) patients receiving natalizumab in a clinical trial. This monoclonal antibody to α4 integrins blocks inflammatory cell entry into the brain and blocked MS related clinical relapses. The occurrence of two very different demyelinating diseases in the brain of a single patient was unanticipated since PML and MS have very little in common except the destruction of myelin. PML is a viral induced lytic brain infection while the etiology of relapsing/remitting MS is an autoimmune response. PML in these MS patients was quickly associated with natalizumab, primarily because MS patients had been treated with other immune therapies for decades without reports of PML.[1-4] Subsequently, there have been several rare reports of PML in MS patients on other therapies like dimethyl fumarates and fingolimod. [5-9] The initial incidence of PML in natalizumab treated MS patients in the phase 3 trial was estimated to be 1 in 1000.[4] Ten years later, >750 PML cases have been reported with >20% fatality rate, and substantial morbidity to survivors. (Biogen, Tysabri Safety Update, September 2017, https://medinfo.biogen.com/secure/pmlresource) The incidence in patients on >24 months treatment, antibody evidence of JC Virus (JCV) exposure, and prior immunosuppressant treatment has reached at least 1 in 70, an incidence much higher than any other opportunistic infection in this setting.,[10,11] Use of a more quantitative antibody index has recently yielded estimates of 2.7% after 72 months natalizumab therapy with prior immunosuppressant exposure.[12] The incidence of PML in MS patients on other immune modulating therapies is much less, perhaps 1 in 10,000 to 1 in 100,000. So what have we learned from this experience on the pathogenesis of PML and how might that knowledge be applied to distinguishing therapy associated risks of PML that would help establish evidenced based monitoring of patients and inform the selection of effective MS treatments for individual patients? There are several areas that surfaced from investigations on PML in MS patients that will be explored in this review: 1. The molecular aspects of pathogenesis and cell specific involvement of JCV infection leading to PML, that are generally applicable to all

PML cases regardless of various underlying diseases. 2. the central role of magnetic resonance imaging (MRI) in the diagnosis of PML, monitoring treated patients to control its morbidity and advancing our understanding of aspects of its pathophysiology, and 3. new insights of clinical value of early PML detection.

JC Virus infection and PML pathogenesis

To understand the complexities of the pathogenesis of PML, it is important to detail some of the background and biology of JCV infection leading to PML. First described in 1958, PML is usually characterized as a rare disease caused by JC Virus, named from the initials of the first patient from whom the virus was isolated in 1971.[13] PML develops in patients with compromised immune systems, particularly cell mediated immune responses. Until the mid 1980s, PML was reported in patients with underlying neoplastic diseases, mostly lymphoproliferative diseases, and a few organ transplant patients treated with immune suppression for graft protection.[14] In the mid- 1980s, HIV-1 infection became the predominate risk with up to 5% of AIDS deaths associated with PML. Effective antiretroviral therapy and earlier initiation to avert severe immunodeficiency have decreased the risk in HIV infected patients to <1% . [15,16] Using a Pubmed search in 2017, we found that since 2005, reports of PML in patients with MS and other underlying diseases, and therapies to treat them, have increased 10 fold suggesting a greater awareness of PML based on clinical evaluation, MRI imaging and use of laboratory tests for JCV DNA and anti JCV antibody. It may be time now to consider PML not as just a rare disease but as a substantial neurological complication in certain high risk populations.

The pathophysiology of JCV in human hosts leading to PML is outlined in Figure 1 following steps 1 through 10 with additional details in Table 1, further annotated in Table 1. JCV has a narrow cellular host range and a variable effect on the organs it infects. Infection in human endothelial cells in the kidney[17-20] and in cells of hematopoietic lineage like CD34+, B cell phenotypes, CD19+ and CD20+ have little pathological effect making infection of hosts a silent event.[21] In the brain however, the multiplication in

oligodendrocytes is lytic and results in PML with devastating clinical consequences. Infection of the neurons in granular cell layer in the cerebellum can also result in a symptomatic neuronopathy. [22]

For cells to be susceptible to JCV infection, they need to express DNA binding proteins that recognize the viral genome, non-coding control region (NCCR) that initiates viral DNA replication and transcription for RNA and eventually protein synthesis (Figure 1steps 6,7/Table 1). There are a number of such transcription factors that are critical to JCV multiplication. The noncoding control region (NCCR) nucleotide sequences are represented in two arrangements. The **archetype** NCCR is comprised of approximately 200 linear nucleotides in virions excreted in the urine, figure 1,step 2,which occurs in about 30% of the population. This 'archetype' variant is generally considered non-pathogenic in kidney or, if identified, in other compartments like plasma/serum and even in brain. Virus isolated from PML patient's brain, like the index patient JC, became known as the **prototype** variant associated with pathogenic PML brain and CSF.[23] These approximately 200 NCCR nucleotides are not arranged linearly but in direct tandem repeats of 98 nucleotide base pairs or other arrangements but always showing duplications. It is thought that the prototype variant is derived from the archetype by deletion and duplication. The tissue compartment or cell type in which a 'rearrangement' of the NCCR from archetype to prototype could take place is still not known but lymphoid cells are a very probable host. (Fig 1, steps 4,5)[24-27] While there are no specific studies in cell culture or in patients that show that such a rearrangement can take place, there are very compelling indirect data supporting such a mechanism. New evidence even implicates Epstein Barr virus coinfection as possible catalyst in the nucleotide transition of the archetype to the prototype variants.[28]

Sero-epidemiological studies show a global distribution of JCV with an estimated rate greater than 50% of the adult population having been exposed. [29]The initial site of infection is still not known but thought to be ingestion or perhaps respiratory inhalation. (Figure 1, step1,) Contact with JCV most commonly results in a subclinical infection during which individuals develop antibodies and cell mediated immune responses (Table 1).[30,31] The serology test used for these studies had been hemagglutination inhibition(HI) based on the virions' ability to aggregate human type O erythrocytes. Anti JCV antibody would inhibit that reaction. Using this assay, a recent

epidemiological study showed a correlation of the percent of the population by decade of age, from 15% in juvenile ages to 80% in the 7th and 8th decade.[32] This observation has been verified with the development of ELISA assays that use recombinant produced VP-1 and not whole virions.[33] VP-1 is the major capsid protein making the outer structure of the viral icosahedral particle and functions in cell attachment. The commercial Quest Diagnostic assay, Stratify™ JCV and the next generation Stratify JCV™ Dx Select™ uses the same VP-1 produced by recombinant technology.[34] Based on these assays, approximately 55% of MS patients are JCV seropositive. There are patients who 'seroconvert' from negative to positive at a higher rate than those who 'serorevert'. The rates of seroconversion in either direction may range from 3% to as high as 10% over a course of years.[35] This observation can complicate testing for anti JCV antibody as part of a risk mitigation program for PML since presence of antibody indicates prior viral infection. An increase in antibody titer or index indicates a history of active infection resulting from a persistent or reactivation of latent infection. An algorithm using the antibody index, calculated using the optical density in the Stratify ELISA assay, shows a correlation with PML risk.[36] A substantial rise in antibody has been identified in PML patients both in their plasma and CSF for weeks or months after diagnosis.[37] There are also reports of an increase in antibody to JCV in natalizumab treated MS patients that is most likely due to release of a previous latent or persistent JCV infection with a subsequent antibody response. The value of regular intervals of anti JCV antibody monitoring is important but should be put into context. The antibody results indicate whether a patient has been exposed and if titers/index increases substantially may indicate that an active infection has taken place. However, reliance on antibody titers/index change is limited as illustrated by the apparent absence of increased titers in patients with prior immunosuppression. However, fundamental virology principles across most viruses that can become latent accept that individuals who have been exposed to a virus are at greater risk for disease from that virus than those who have not been.

In addition, anti JCV antibodies may be directed to different regions of the primary capsid protein, VP-1, that could be unique to a particular patient. As is the situation with the viral NCCR, the VP-1 gene can be hypervariable producing a number of VP-1 proteins with different primary amino acid sequences compared with the prototype variant. This has been known for many years in the thorough description of the 'JCV Type' linking geographical locations with independent VP -1 genes and protein variants.[38] So it is

not surprising that any one PML patient may have multiple representations of VP-1 protein at any one time. This observation had led to the hypothesis of VP-1 gene rearrangement that could result in a more 'neurovirulent' variant leading to PML.[39,40] This observation warrants further investigation that would require 'deep sequencing' studies.[41] However, it appears that PML patients are infected with the prototype VP-1 protein since that is the antigen used in the ELISA assays of commercial, academic and government laboratories. It is possible that immune escape of JCV VP-1 variants could occur due to either persistent JCV in cell compartments or mutations in the VP-1 gene to avoid immune recognition.[40] This has been an area of new investigation in the last several years.

However, antibodies to JCV may not result in protection against PML development (and are thus not necessarily neutralizing antibodies). There is ample in vitro data showing antibody made against JCV blocks virion adsorption to target cells that limits attachment and entry, thus reducing viral multiplication. But there is little clinical evidence in healthy people or patients showing antibody may help control JCV infection.[42] In fact nearly all individuals who persistently shed JCV in their urine are seropositive. Some seropositive individuals can even be viremic, and PML patients can have very high levels of CSF antibody in the presence of high viral DNA copy numbers.[43,44] Consequently, CD4+ and CD8+ cytotoxic cell recognition of viral antigens probably play a more significant role against JCV infection. CD8+ cytotoxic T cells to JCV prototype VP-1 have been identified in PML and non-PML patients for many years.[45,46] (Fig 1,step7) With the increase reported incidence of PML in MS patients treated with DMTs, newer studies have identified CD 4+ T cells as critical to control of JCV in natalizumab treated patients directed against the 4 major JCV proteins, T antigen, VP-1, VP-2, and agno. In addition, lack of CD 4+ cells and those releasing Il-10 were identified in natalizumab treated MS patients including one of the index cases who remained persistently JCV positive in CSF for years.[47] CD 4+ T cells have also been cultured from brain tissue of PML patients that are directed to potential more neurotropic viral capsid proteins not identified by CD 4+ T cells in the periphery. These CD 4+ cells seemed necessary to stimulate cytotoxic CD8+ cells to function for clearance of JCV from the brain so perhaps were lacking in PML patients. These laboratory and clinical studies have been undertaken directly in response to the need for a better understanding of the immune system role because of PML incidence in MS patients. Perhaps in further defining risk factors for PML, identifying CD 4+, CD8+ and other immune system cells for activity to JCV antigens would be

informative in the PML high risk patients. Unlike MS, the specific etiologic cause of PML is well known, JC Virus lytic infection of oligodendrocytes. To acquire a deeper understanding of the pathogenesis of PML, there needs to be a magnified focus on the virus including the stages of infection leading to oligodendrocyte cell death. (Figure 1, step 10)

A case can be made that common pathophysiologic pathways explain the steps leading to PML, regardless of the underlying risk that allowed it. For example, some patients with T cell immune compromised systems may harbor JCV in a latent state in tissues like kidney, lymphoid organs like bone marrow, and possibly brain. Periodic JCV release from latency or even a persistent infection is poorly managed by the immune system so virus may enter the brain as free virions or through an infected cell (Figure 1, step 9/Table 1). CD 4+ cells that do not adequately recognize JCV antigens have now become an important part of lack of immune surveillance[48] while cells in the B cell lineage have been implicated as possible carriers since JCV has been identified in CD 19 and CD 20 cells.[49] The brain is not the initial site of JCV infection and data on latency in brain are very limited. There have been reports of identification of JCV DNA in brain tissues of non-PML patients.[50] In these reports, there was no evidence that the entire viral genome was present in order to initiate and sustain viral multiplication. There is only one study that specifically investigated the presence of JCV DNA in MS brain tissue and found it absent.[51] A multicenter study using blinded samples and controls of positive and negative brain tissues should be considered to determine the existence of latent JCV in the brain. However, at this point it is more likely that release of latent JCV in the periphery, particularly the virulent variant, is a key factor. The kidney/urine derived variant is considered non-virulent or at least less neurotropic, so the best candidate for latency is likely in lymphoid cells (Fig 1, step 4,5,Table 1). [52]These cells can be hosts for rearrangement of the viral NCCR and perhaps gene rearrangement of the VP-1. They would be subject to factors that activate viruses like EBV that may even assist in JCV NCCR rearrangement from the archetype to the prototype by gene rearrangement and insertion as well as the potential to be targets for RAG 1 and RAG2 enzymatic mechanisms best known for their role in immunogloblulin diversity.[53,54]

So a question becomes what unique features does natalizumab possess that no other therapy associated PML risk shares. Natalizumab associated PML patients are not systemically immune suppressed. Other opportunistic infections are not prominent, suggesting PML is a specifically enhanced problem rather than the result of broad immunosuppression. Further, it appears to require years for the risk to be manifest. These two factors highlight the need to understand PML pathogenesis beyond pure immune suppressive explanations. It may be over simplistic to suggest that lack of immune surveillance is the major underlying mechanism of PML in natalizumab treated MS patients. Even with immune reconstitution inflammatory syndrome (IRIS), some natalizumab treated PML patients continue to have detectable virus in CSF for months to years.[37] We see two unique features of natalizumab that contribute to its special risk. One is that natalizumab forces migration of hematopioietic stem cells, CD34+ and precursors of B cells from the bone marrow (Figure 1, step 4). It shares this feature with efalizumab, the other monoclonal with highest risk of PML. The other is the temporal relationship of PML incidence after long term dosing, approximately 2 years or longer. JCV can be latent/persistent in CD 34+ or preB cells in the bone marrow described by several laboratories[24,25,55] and in culture models identifying DNA binding factors that act on the JCV transcription sites.[56] These factors can also be found in CD 19 and CD 20 cells in the peripheral circulation. It is possible that the high percent of such cells forced out of the bone marrow for long periods would result in release of some latently infected cells (Figure 1, step 5). In those individuals, perhaps their immune system cells do not completely clear newly released virions particularly if remaining intracellular like EBV. But that observation does not account for the temporal correlation of the high incidence of PML after nearly 2 years of dosing. However, natalizumab also upregulates genes in a critical pathway for maturation of B cells, POU domain DNA transcription factors particularly Spi B that binds JCV NCCR. The time course of natalizumab effect on POU domain regulation is consistent with PML incidence. [57,58] The two characteristics only occurring in natalizumab, forced migration of cells from the bone marrow and temporal upregulation of factors that highly favor JCV growth match the current observations of delayed PML incidence and focuses attention on the cause of PML, JC Virus cellular interactions leading to PML (Table 1, steps 5,6). While perhaps still premature, it is noteworthy to consider how laboratory analysis of these factors in immune

cells as well as immune cell antiviral function would further identify PML high risk patients before oligodendrocyte infection is initiated.[59] [60]

MRI Imaging for Early PML Detection/Diagnosis/Management

The approach to diagnosis of PML has been reviewed elsewhere, but routinely requires identification of active CNS pathology and JC virus in the brain.[61] Brain imaging is a critical contributor to the diagnosis of PML.[61] Indeed, without an MRI lesion, PML diagnosis cannot be verified. The sensitivity of MRI in identifying PML lesions has made it the modality of choice in monitoring natalizumab treated MS patients for early detection of PML. Consideration of imaging in relation to the clinical stages of PML requires understanding the clinical manifestations that PML takes, depending on the degree of brain infection, as well as the status of immune response to this unique infection. (Table 2) We define onset of PML as the time JC virus enters brain and infects oligodendrocytes, which ultimately leads to a clinical serious brain injury. Table 2 emphasizes that there is a pre-symptomatic period during which the infection grows which even by MRI is likely to be 3-6 months in duration.[62] This accounts for some of the low risk of early months of therapy, as well as the interval when PML is most likely to be seen after stopping natalizumab and transitioning to a low risk therapy. The symptomatic disease state is very different depending on whether immune reconstitution is achieved or not. Without immune reconstitution, the "classic" PML is generally fatal, and no effective immune response is generated. Alternatively, as generally occurs in natalizumab cases, successful immune reconstitution precipitates an inflammatory syndrome that can arrest the disease. This response must come quickly enough to avert death from disease progression, but when it occurs and the patient survives >6 months, the viral disease is generally controlled, albeit with a fixed brain lesion seen in post-PML survivors. (Table 2) PML therapy has been reviewed in detail elsewhere.[63] No anti-viral therapies, including widely used mirtazapine and mefloquine[64], have been demonstrated to improve outcomes, but it is abundantly clear that immune reconstitution changes the course of PML for the better. The concept of using plasma exchange to hasten immune reconstitution with natalizumab cases is thus a rational approach that

has been widely adopted and associated with PML outcomes that outpace historical precedents.[65] However, the balance of concerns about potential augmentation of damaging IRIS remains a concern that clinicians must balance.[66,67] Similarly, active use of corticosteroids or maraviroc[68] to blunt IRIS remain controversial, but at least in more advanced disease active immune reconstitution seems likely to contribute to better outcomes. Gathering informative data to more clearly articulate recommendations remains extremely challenging with this rare and serious disease. Urgency for early diagnosis of PML (Table 2, 3), preferably before the onset of clinical symptoms, aims at limiting brain damage and thus disability. Recommended MR parameters are widely available. Increasingly, annual scans including brain and spinal cord are recommended to monitor the efficacy of DMT for MS (Table 3). Even more frequent scans of brain alone are recommended seeking early detection of PML in higher risk settings. Retrospective analysis of some PML patients with frequent scans demonstrates lesions developing months before symptoms.[62] It is now recognized that development of PML symptoms may only occur months after JCV enters the brain and forms a visible lesion with MRI. To date, we found 19 publications [2,62,69-83] reporting on 48 PML patients asymptomatic at the time of a detectable lesion. Twenty-one of these patients developed symptoms in up to 41 weeks after lesion visualization and in a further 13 patients natalizumab was withdrawn before the development of symptoms, with 4 patients remaining symptom free. Disabling outcomes including mortality appear to be reduced in these patients.[82]

It is critical to be aware that verified PML lesions actively evolve on repeated imaging, either because the JCV induced disease progresses, or because the inflammatory response controlling the infection results in evolution of the image characteristics. Thus, repeated MRI images that do not change help rule out PML, while evolving lesions are consistent with a PML diagnosis. PML may not be diagnosed on a single MRI without additional clinical and virological confirmation.

Despite the increasing number of PML cases reported, the low frequency, sporadic appearance, and uncontrolled clinical market status of natalizumab distribution, make a prospective assessment of the sensitivity, specificity and accuracy of imaging difficult. It has been suggested that the 4 most helpful features suggesting a PML lesion (applicable to lesions in asymptomatic patients) are its subcortical location (involvement of U-fibers), T1 hypointensity, DW hyperintensity, and the presence of punctate T2-

hyperintense lesions.[80,84] (Figure 2) Unlike AIDS associated PML, GD contrast enhancement is often seen even at presentation in PML in the setting of treated MS. Occasional cortical and deep GM involvement can occur but white matter distribution dominates PML.

The punctate lesions may offer some insight into the pathophysiology of PML, suggesting an inflammatory response in the lesion. Recognition of this imaging pattern has emerged in settings where partial immune response to JC virus is commonly present, and was not noted in the era when most cases were AIDS associated and lacked inflammatory response on pathologic exam. Punctate lesions appear to develop in perivascular spaces in the brain, where JCV in mononuclear cells and infected glial cells has been identified.[25] Histological examination has shown that inflammation typical of IRIS to JC virus is associated with a marked infiltration of CD8+ T lymphocytes, especially in the perivascular spaces.[85] The frequent observation of this pattern thus may be a marker of IRIS, and is consistent with the early evidence of contrast enhancement suggesting IRIS in many natalizumab associated cases of PML. While punctate lesions often enhance with GD, their unenhanced presence on T1suggests pathology outside intrinsic brain cells supporting JCV replication, and thus may instead specifically reflect inflammatory response. The alternative interpretation that these are the smallest "islands" of demyelination in early infection is plausible, but their early enhancement favors their location in relation to blood vessels with increased permeability to GD. If these lesions reliably represent disease with IRIS, they could direct clinicians to focus on anti-inflammatory therapy for these patients. Another interesting MRI lesion similarly reflecting probable inflammatory responses is a T1 bright subcortical lesion that is often associated with seizures and inflammatory PML lesions.[86](Figure 2)

Confirming the Diagnosis of Pre-symptomatic PML

The success of frequent MRI brain imaging will be measured by the identification of increased proportion of asymptomatic lesions determined to be PML. AAN Diagnostic Criteria[61] require symptoms for definite diagnosis, yet ideally PML would be detected and arrested without symptomatic brain damage occurring through close MRI monitoring of high risk patients. Verification of a PML diagnosis without symptoms is challenging. Very early, CSF viral load may be low or undetectable and the dynamic nature of PML

cannot be confirmed by a single scan. MRI lesions may be characteristic of PML, but no MRI features have been described as being pathognomonic. Small lesions can be difficult to differentiate from MS lesions especially when there is a high lesion load.[83]

A critical clinical point is that in patients at risk, new MRI lesions consistent with PML should be assumed to be PML, and active longitudinal diagnostic and therapeutic steps including repeated CSF sampling (if required), repeated MRI imaging, and serial JCV antibody titers should be performed to help establish the diagnosis. During these procedures clinical management should be pursued as if PML is present. Delay in managing PML by awaiting AAN definite diagnostic criteria, would sacrifice the benefits of early detection gained by monitoring with MRI. Such an approach was successfully implemented in at least 3 patients with PML compatible MRI changes but negative CSF JCV PCR.[69,71] In these 3 patients managed as if the diagnosis was established, two had subsequent detection of JCV in CSF on repeat sampling. In all patients the MR imaging evolved to a pattern compatible with development of PML with IRIS, helping to strongly support the diagnosis. Often asymptomatic patients later develop symptoms associated with IRIS, ultimately fulfilling traditional diagnostic criteria.

To date, serial quantitative JCV antibody determinations have too rarely been used to help consideration of possible PML in difficult cases. Active JC virus disease including PML typically drives an increase in JCV antibody titers that confirms JCV related disease. Thus, even if viral DNA is not demonstrated in CSF, if compatible and evolving MRI lesions are associated with increasing systemic JCV antibody titers, this should provide significant support for diagnosis of PML.[87] Use of this approach may not work in the face of prior immunotherapy, however, necessitating biopsy or presumptive diagnosis without confirmation. Brain biopsy remains the ultimate criteria when a definite diagnosis is required lacking detection of viral DNA in the CSF. However, with small pre-clinical lesions, it will be difficult to biopsy at the earliest stages, and should be only used judiciously when certainty about the diagnosis is clinically critical.

Risk Mitigation Strategies Are Failing Us

A risk mitigation strategy was developed by Biogen/Idec to protect patients from developing PML in the setting of natalizumab therapy.[88] The fundamentals have been actively discussed, and variably applied. [10,11,89-93] However, the ideal of witnessing plummeting incidence of PML cases has not yet materialized. [94] We summarize our own suggestions based on a most recent algorithm [95] and the data we are aware of in Table 3. We propose the surveillance be guided by the estimated risk, dichotomizing it into 2 groups: a) regular surveillance if the PML risk is $\leq$ 0.9/1000 and b) intensive surveillance if it is above 0.9/1000 patients. This approach allows simple adjustments when the estimated risks change or new risks are identified. (Insert: Recommendations)

Shortcomings of risk stratification elements

The substance of the three key risk stratification elements is known to have flaws that might help understand the suboptimal impact they exert. First, while JCV antibody is a predictor substantiating infection with the virus causing the disease. Unfortunately, JCV viremia and viruria can be present in antibody negative patients.[96] Further, quantitative antibody analysis, while suggestive of more active infection with higher risk, fails to be predictive after prior immunotherapy. While overall expression of antibodies inversely correlates with disease risk, some evidence that antibodies still may play a role in controlling this virus is emerging, reviving interest in vaccination strategies for JC virus or PML management.[42,97] Thus, JCV antibody status falls far short of an ideal biomarker. Second, duration of therapy as a risk parameter is also flawed. The measured variable is duration of time from natalizumab therapy start to clinical diagnosis of PML, which itself may be a considerable time after the first symptoms.[98] The actual biologic interval of interest is time to brain infection with the virus. Through observations with more intense monitoring of pre-symptomatic high risk populations, we now realize that infection likely takes place at least 6 months prior to the clinical manifestations of disease, substantiated by observation of pre-symptomatic lesions of PML on MRI scans, and by pre-symptomatic immunoglobulin elevations leading up to PML diagnosis.[29,82] It is likely that the pre-symptomatic interval is even more variable related to the eloquence of clinical expression of lesions in different brain regions. For example, it seems likely that brainstem lesions would more rapidly lead to symptoms compared with frontal lobe lesions. Thus, extrapolation about specifics of pathophysiology based on the crude interval from

start of therapy to clinically symptomatic disease is quite imprecise. Recent critical analysis about the imprecision of the Biogen risk estimates for impact of duration of infection become even less meaningful, when the imprecision of biology reflected by the measure is considered more critically.[92] Third and finally, the impact of prior immune suppression on risk is similarly quite poorly fleshed out in literature. It is fundamentally untenable that the specifics of type and duration of prior immunotherapy is of little consequence in determining risk on a biologic basis, yet this is at present a monolithic consideration. A dose of azathioprine would receive equal weight with long term cyclophosphamide therapy, yet impact on the immune system must be very different.

Thus, current negative commentary on the precision of the present risk mitigation strategies is unsurprising, but perhaps clinically not so critical.[10,92,99,100]

Risk stratification with newer disease modifying MS therapies

The risk mitigation developed for natalizumab is likely only truly applicable in relation to that drug. PML risk with other available and emerging DMT for MS (dimethyl fumarate, fingolimod, rituximab, ocrelizumab) is much lower,[89] and while its presence must be acknowledged, it should not severely impact decision making where benefits can be accrued by implementing early and effective MS therapy. In the case of dimethyl fumarate, monitoring for lymphopenia appears likely to identify a higher risk group in whom alternate therapy should be sought. In that setting prolonged lymphopenia with absolute lymphocyte counts <750 accounts for most cases, although the risk may reside particularly in the loss of CD8 cells critical to JCV control.[101] Measurement of circulating lymphopenia however is not universally helpful. For fingolimod, this strategy cannot be applied since circulating lymphocytes decline while effective lymphocytic function appears largely normal. Similarly, alemtuzumab associated risk for PML has not been demonstrated in MS patients yet despite marked impact on lymphocyte profiles. Alternatives to lymphocyte counts might include serial antibody measurements, or monitoring for circulating JC virus. The multiplex PCR that allows identification of emergence of

prototypic virus that likely has enhanced risk of PML seems a plausible means of risk stratification, but has not yet been demonstrated to serve in this way.[23] However, the low overall risk with alternative DMT makes it difficult to validate, and probably impractical to use as a stratification factor in practice. Other alternative PML risk stratification approaches under investigation in natalizumab associated PML include measurements of CD62L and lipid-specific IgM bands. [102,103]At present similar logic applies to rituximab and ocrelizumab. These monoclonal antibodies directed against B cells have yet to demonstrate excess risk of PML in MS patients despite a large number of cases associated with rituximab when used in the setting of hematologic malignancies and other diseases with greater underlying risk of PML.[104,105] The theoretical risk suggests clinical vigilance, but no other risk mitigating strategy can be recommended for PML at this time when using these emerging MS therapies.

Thoughts on risk mediation based on what has been learned

Ongoing consideration of additional risk mitigating factors that would aid risk assessment and be more predictive should be a theme of investigation. Technology that allows more detailed consideration of JCV specific immune control might more accurately reflect risk. Quantitative definition of the specifics of T cell recognition and response, as well as identification of the emergence of prototypic virus might well alert the clinician to a small subset of high risk patients in whom therapy would be foolhardy.

The fact that the present system has largely deluded us all is evidenced by the lack of impact so far on the incidence of new cases. While the imprecision of the present risk model is likely in part to blame, the most likely cause is that risk monitoring and communication is either too inconsistently done to inform patients, or that they are choosing to continue to use natalizumab even when they have a significantly elevated risk.

Development of therapeutics for auto-immune diseases including MS, genetic origin of immune disorders or neoplastic disease is in evolution, and optimizing these choices to include PML risk will require more detailed data than currently exists. For example, the relative efficacy of MS therapies, as well as their costs must inform prescribing patterns. Estimates of these factors are difficult to

substantiate. These factors must be integrated with the risk of PML encumbered by various therapies. Calculation of all of these factors, and explaining them to a patient who must fit this evidence into a personal risk tolerance profile is a very difficult task. Better tools need to be developed to assist patients and physicians in meaningful ways to understand this and come to an ethically sound decision for the patient's management.[106]

Conclusion and future directions

The past decade has witnessed substantial progress in understanding JC virus and PML. The close observation and additional cases seen in multiple sclerosis patients has given the opportunity to enrich the molecular biology of JC virus, and to make some progress on likely evolution of risk and invasion of the brain. Enhanced identification of higher risk patients has allowed the evolution of use of MRI, such that detection of PML lesions prior to symptom onset is commonplace in high risk patients. Improved use and interpretation of MRI have proved pivotal for PML. However, the clinical management of MS patients remains challenging.

Further, the outcomes from PML have markedly improved. While PML is still a serious and sometimes lethal disease, a majority of patients contracting it survive in settings where immune reconstitution is possible, and with early detection of disease, commonly severe disability from PML can be avoided. However, we still are unable to detect individual risk precisely enough to give easy instructions about PML, and still settle for very early diagnosis to minimize injury.

Meanwhile, the practical means to enhance communication about risk and help patients select the optimal approach to their illness tailored by their own willingness to take risk is an ongoing clinical challenge. It is especially important to be sure that it is not for lack of monitoring and acceptance of known risk that patients are developing PML. If on the other hand, patients have accepted the risks and continued therapy with full knowledge of risks and benefits, principles of ethical care have been served. Ultimately understanding

the overall difference in outcomes of those who accept the risk with DMT and do well, compared to those who develop PML, should be understood and have acceptable value, if the choice to use this therapy is to continue to be up to patients and their clinicians.

The basis for such an analysis is the availability of credible data. PML is not a reportable disease, and detailed retrospective data gathering is laborious and incomplete. Registration of cases with systematic reporting of circumstances of the disease would allow us to study the impact of risk mitigation concepts. Development of widespread or universal data collection and consideration of cases could speed research on risk and outcomes, and allow more precise risk mitigation programs. We believe that while the mitigation strategies are not perfect, the largest failure is in not implementing changes in therapy when risk is known to be elevated. With MS therapies that are comparably effective to natalizumab, we believe replacement of natalizumab in high risk patients should be more uniformly employed and should reduce the burden of this tragic disease.

Table 1 – Stages of JCV Infection Leading to PML

TABLE 1:	Urine[a]		Blood[b]		CSF[c]	
	Antibody[d]	DNA[e]	Antibody	DNA	Antibody	DNA
A. Primary Infection[30, 31] (Ingestion or primary inhalation)	Not measured	Not symptomatic so rarely measured	Emerging in juvenile years (15%) increasing to 80% in 7th and 8th decade) Seroconversion 3-10%	Not measured	Not measured	Not measured
B. Establish Latency in kidney [17-20]	Not measured	Can be undetected to low or $>10^{6/7}$c/ml; sporadic or continual release	Variable levels from low to high titers	Can be transiently detected generally $<10^2$ c/ml	Not present	Not present
C. Escape from kidney to circulation; may enter lymphoid organs like bone marrow[21,24,26,27,53,56]	Not measured	Can be undetected to low or $>10^{6/7}$c/ml; sporadic or continual release	Variable levels, % population seropositive increases with age	Can be transiently detected generally $<10^2$ c/ml	Not present	Not present
D. Escape into circulation, in cells or free virions enters brain, infects oligodendrocytes[28,32,48,49,57]	Not measured	Can be undetected to low or $>10^{6/7}$c/ml;	Detected at variable levels, titer increase during onset of PML	Transiently detectable, variable levels commonly 50 to > 500 c/ml	High titer or index indicating intrathecal antibody production	Generally detectable at 10-10^7c/ml

Footnotes for Table 1

a. ***Urine samples*** can be tested for JCV DNA to demonstrate a latent or persistent infection. Approximately 30% or more of the population globally excrete JC Virus in the urine, viruria, without pathological effects in the kidney although very high levels of viral DNA can be present. The JCV ***archetype*** variant, unique arrangement of non-repeating nucleotide sequences in the non-coding control region or NCCR, is the most common in the urine. The JCV archetype variant is thought not to be neurotropic and is rarely detected in brain or CSF of PML patients.

b.Blood samples are tested for antibody to JCV in either serum or plasma indicating prior exposure to JCV. Seroepidemiology of JCV has shown a global presence of JC Virus infection. However, multiple alterations throughout the genome can be found that have used to 'Type' JCV allowing studies to define geographical distribution and follow transmission in family members. High levels of antibody or increasing levels of antibody, reported as a titer or index, usually indicate active infection from reactivation of latency or a new infectious episode. Antibody levels also may fluctuate so sero-positive or sero-negative status may change over time. Estimates that this conversion takes place in 2% to greater than 10% of the population annually. Also, rare seronegative individuals may experience JCV infection and not show or make antibody as evidenced by viruia or viremia.[43]

Blood samples are also used to test cell compartments for JCV DNA as cell carriers for infection or persistence. Notably viral DNA has been found in CD19+/20+, CD34+ cells but not in CD 3 (T cells) or monocytes. [21]

c. ***CSF samples*** with detectable JCV DNA serve as the laboratory confirmation of PML diagnosis. Quantification of viral DNA is reported as genome copies per ml. Currently, the most sensitive assay has a limit of detection of 10 c/ml.[23] Usually, the lower the copy number of viral DNA the better the PML prognosis. Viral DNA in the CSF is the ***prototype*** variant with repeat nucleotide sequences in the NCCR thought to be derived from the ***archetype*** variant through deletion, duplication and rearrangement. This transformation from the archetype to prototype probably takes place before entry into the brain in lymphoid tissues like nodes or bone marrow.[52,53] There can be multiple nucleotide arrangements of the NCCR in PML patients plasma, brain and CSF. However, generally no two PML patients demonstrate identical patterns although the same variant is found throughout an individual PML patient's tissues. [41]

CSF samples also can be tested for intrathecal antibody to JCV that occurs in PML patients and may be used as a marker or sign of developing PML.[43]

d***. Antibody*** is measured by ELISA assay using viral major capsid protein, VP1 derived from the prototype variant, as antigen; result reported as titer [33]or index[34], depending on assay, reflecting the level of antibody response, usually IgG. Other VP1 variants have been reported that may have escaped immune control with mutations in the VP1 sequences that were identified in the CSF of PML patients with specific T cell responses so attributed as the potential PML causative variant.[40] However, almost all PML patients with ELISA demonstrated antibody to JCV have the prototype variant in brain and CSF.

e. Viral DNA is measured using quantitative polymerase chain reaction specifically measuring copy number of viral genome DNA as c/ml; low=50c/ml; hi=>500c/ml. In MS/PML patients, the median c/ml is >100c/ml to <500 c/ml in a range of 10c/ml to 10^7c/ml [37] Primary infection is subacute, can occur in infancy from parental transfer to early juvenile years as determined by antibody presence.

Table 2: Stages of Progressive Multifocal Leukoencephalopathy

	Pathology	Duration	Blood		CSF		MRI
			Antibody	DNA	Antibody	DNA	
A. Pre-symptomatic PML	Unknown, likely as in "Classic" below	3-6 months estimated from viral entry to brain until neurologic sx[1]	Increases, dynamic increase supports PML dx	Transient, 50 to 500 c/ml	Detectable, titer increasing	Generally low titer detectable, 10-10^7 c/ml	New Lesion on surveillance MRI, generally small, DWI bright
B1. "Classical" Symptomatic PML without immune responses[3]	Demyelinating plaques, bizarre astrocytes, oligodendrocytes with nuclear inclusions, notably absent inflammatory response	3-6 months from onset of sx to death if no immune reconstitution	Increases, marked increases typical of PML	Transient, 50 to 500 c/ml	Detectable titer, increasing	10-10^7 c/ml, rarely undetectable	Typical brain lesions[2], enlarging, rare if any CE[4,] no mass effect
B.2PML with immune reconstitution inflammatory syndrome (IRIS) [4]	Classic pattern plus inflammatory response with variable mix of CD8 and CD4 lymphocytes, may have declining amount of JC virus	1-5 months after immune reconstitution[5], associated with potential for survival of PML, may be present at diagnosis in natalizumab PML setting	Increases	Transient, 50 to 500 c/ml	High titer	10-10^7 c/ml, may rise then fall during course	Typical brain lesions[2], CE usual but not required. [4] Punctate pattern and T1 bright cortical line suggest this stage of PML.
C. Post PML survivors[6]	Atrophy, fibrosis, rare JCV infected cells	Years, dependent on underlying disease(s), fixed lesion may support clinical	Little data, but likely relatively stable at high titers	Transient, 50 to 500 c/ml	High Titer	Often undetectable, but may remain detectable[7]	No CE, Brain atrophy in region of prior lesions, Defect with ↑

		improvement 6-12 mo after dx, then is stable clinically in most cases					T1 cortex

[1] Duration of pre-symptomatic stage dependent on location in brain. Clinically silent regions like frontal lobes may harbor pre-symptomatic infection for up to 41 wks in one documented case, while areas causing symptoms with small lesions will be detected in fewer months. Duration of this phase is consistent with symptomatic PML occurring up in the first 6 months after natalizumab discontinuation.

[2]Four most helpful features suggesting a PML lesion in asymptomatic patients: subcortical location (involvement of U-fibers), T1 hypointensity, DW hyperintensity, and the presence of punctate T2-hyperintense lesions. Evolution of lesion on subsequent scans important to substantiating diagnosis.

[3] Typical of PML developing in untreated HIV/AIDS or highly immune deficient setting where virutally no immune response seen. Classically this symptomatic disease led to death within 6 months in most patients.

[4] PML with IRIS can also occur at the pre-symptomatic and symptomatic phase where partial immune deficiency occurs (common in natalizumab MS at onset); punctate lesions (± CE) and T1 cortical bands probably indicate inflammation with or without CE. Prior cortiocsteroids reduce chance of CE without eliminating inflammatory response. CE: Contrast enhancement

[5] PML with IRIS persists for up to 5 months or longer documented by late biopsy of lesions, and may require repeated therapy to suppress.

[6] Deaths from PML typically occur within 6 months of diagnosis, with survivors dying of other causes months to many years later, often hastened by underlying diseases, or the hazard of neurological disability and its complications.

[7] JCV DNA typically declines and often is undetectable after survival of PML. However, virologic cure does not occur, and CSF may continue to have generally low level detectable virus indefinitely.

Table 3: Protocol for PML surveillance in MS patients

Monitoring steps for Patients							MRI Sequences						
Treatment Group	PML risk		S	Anti JCV AB	MRI		Brain					Spine[1]	
				Frequency	Frequency	Indication							
	Treatment Duration	Risk Estimate /1000[a]					FLAIR	T2	DWI	T1	T1+Gd	PD/T2/STIR	T1+Gd
Immunmodulatory treatment					Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
All NTZ patients					Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Anti JCV negative	NA	NA		6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Anti JCV positive, no prior immunosuppression													
Index <0.9	1 – 72M	0.1 – 0.6	**R**	6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Index 0.9-1.5	1 – 36M	0.1 - 0.8	**R**	6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	37 – 72M	*2 - 3*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-
Index >1.5	1 – 24M	0.2 – 0.9	**R**		Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	25 – 72M	*3 - 10*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-
JCV +ve, Previous Immuno-suppression	1-24M	0.3 - 0.4	**R**		Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	25 – 72M	*4 – 8*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-

Abbreviations: FLAIR = fluid-attenuated inversion recovery, DWI = diffusion weighted imaging, I = intensive surveillance, S = surveillance, T1 T1 + Gadolinium, PD = proton density , STIR = Short T1 inversion recovery; NTZ= Natalizumab, R = regular surveillance

[a] Risk estimates from report of EMA, February 2016[107]

MS: Monitor MS activity, justified by aim to provide DMT achieving "no evidence of active disease" (NEAD) for optimal clinical management of MS

PML: PML Surveillance

[1]Spine MRI is not indicated for PML monitoring, but may be used in monitoring MS disease activity, or if neurological exam suggests possible spinal cord localization of pathology

[2]Rovira[108]: **Mandatory** a) Axial proton-density and/or T2-FLAIR/T2-w; b) 2D or 3D contrast-enhanced T1-w

Optional a) Unenhanced 2D or high-resolution isotropic 3D T1w; b) 2D and/or 3D dual inversion recovery; c) Axial DWI

[3]Traboulsee[109]: **Mandatory** (Core) a) Anatomic 3D inversion recovery–prepared T1 gradient echo Gd; b) 3D sagittal T2WI FLAIR; c) 3D T2WI; d) 2D axial DWI; e) 3D FLASH (non-IR prep) postGd

Optional: a) Axial proton attenuation; b) Pre- or post Gd axial T1 spin-echo; c) SWI

[4] Optional in Rovira et al and mandatory in Traboulsee et al

[5] Optional in Rovira et al

[6]McGuigan[95] et al and Traboulsee et al recommend FLAIR

[7]Yousry[83] et al recommend FLAIR or T2-w; there is no data suggesting superiority of one sequence over the other in this specific scenario

[8]Spine Traboulsee et al	Mandatory	a) Sagittal T2; b) Sagittal PD, c) STIR, or PST1-IR; d) Axial T2 through lesions
	Optional	a) Axial T2 through complete cervical cord; Gd & post-Gd sagittal T1
Rovira et al	Mandatory	a) Dual-echo (PD and T2-w); b) SE and/or fast SE; c) STIR (as an alternative to PD-w); d) Gd T1-w SE (if T2 lesions present)
	Optional	a) Phase-sensitive inversion recovery (as an alternative to STIR at the cervical segment)

Axial: a) 2D and/or 3D T2-w fast SE; b)GD T1-w SE

[9]Mandatory in Rovira et al and Optional in Traboulsee et al.

Figure 1

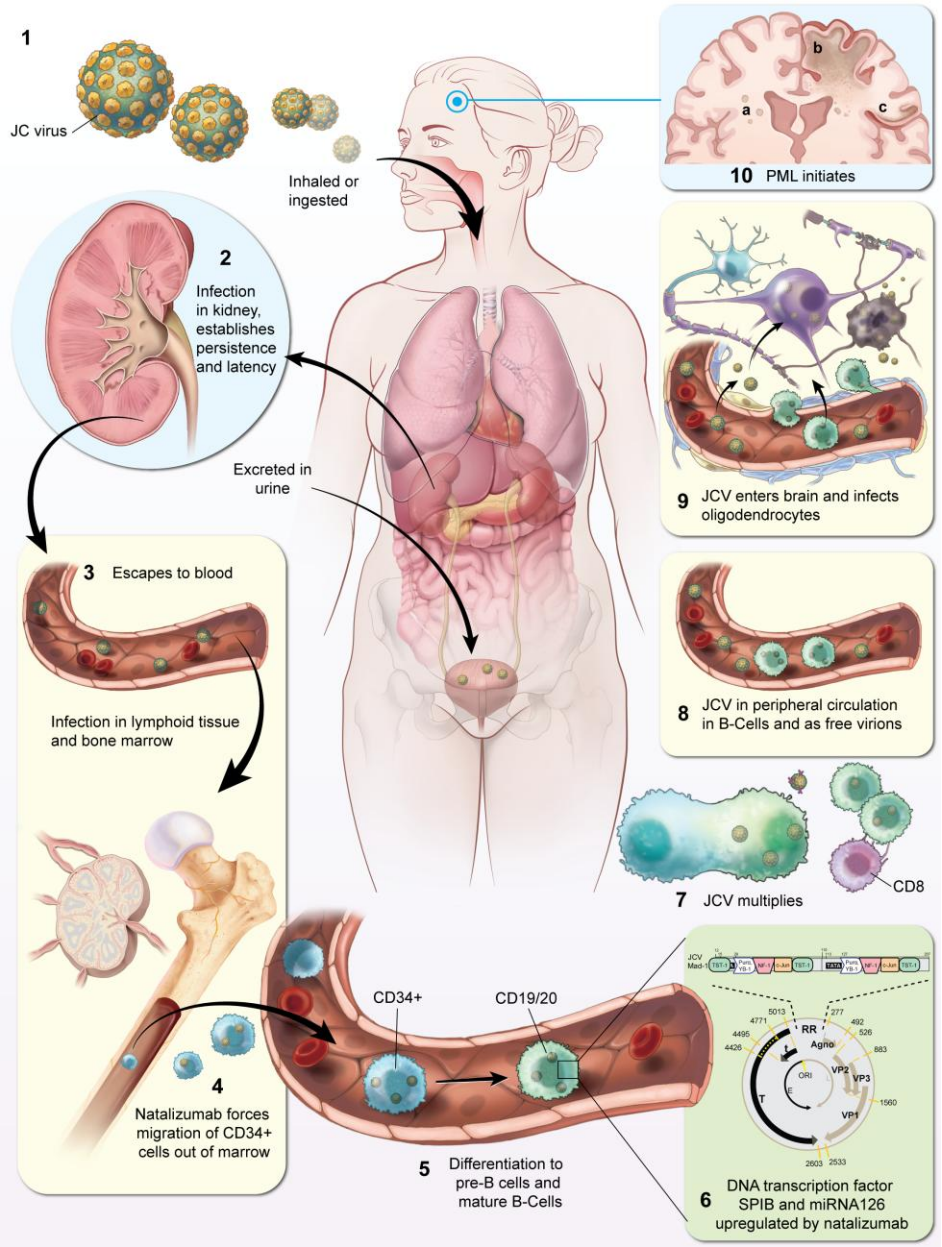
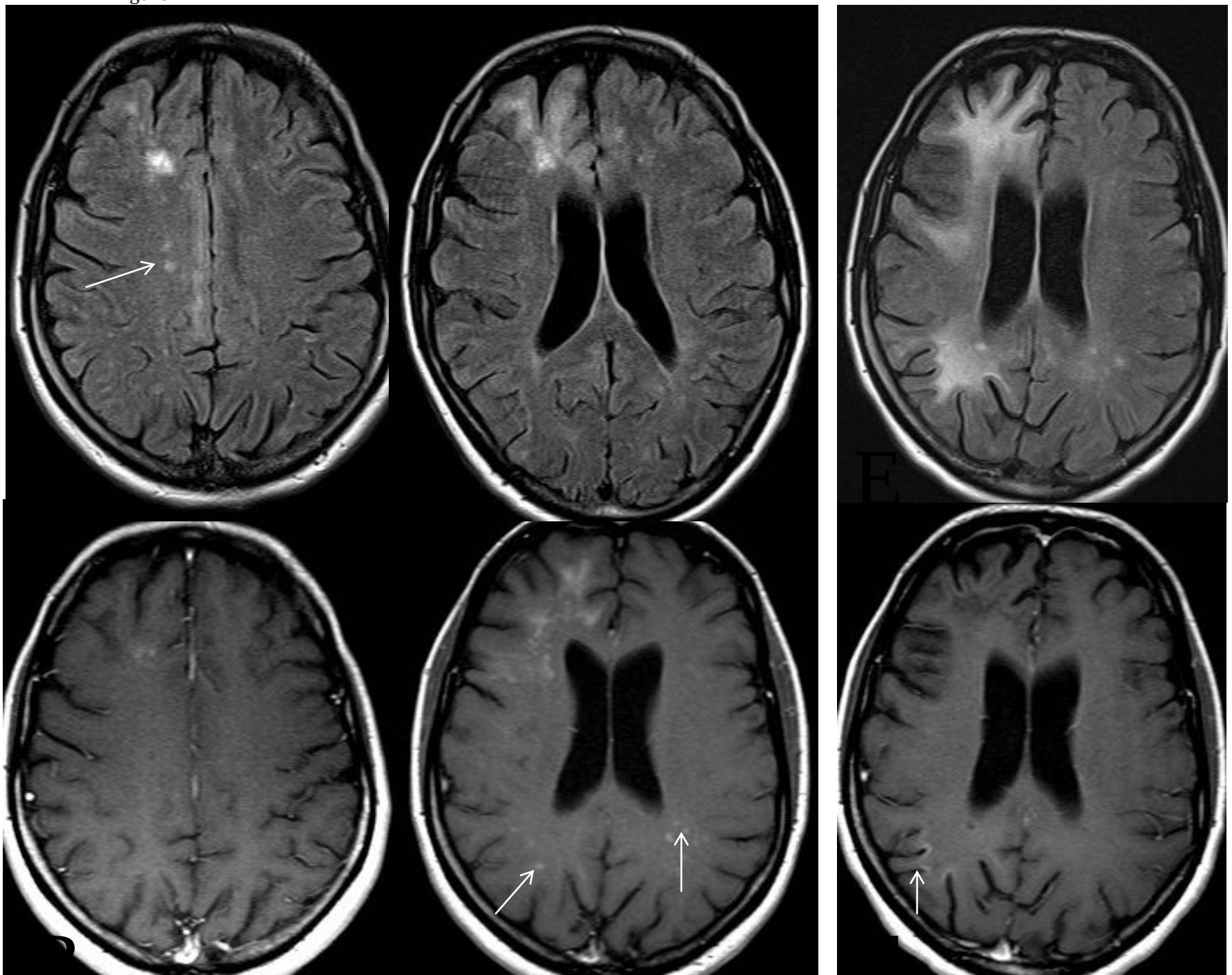

1
JC virus
Inhaled or ingested
2
Infection in kidney, establishes persistence and latency
Excreted in urine
3 Escapes to blood
Infection in lymphoid tissue and bone marrow
4
Natalizumab forces migration of CD34+ cells out of marrow
CD34+
CD19/20
5 Differentiation to pre-B cells and mature B-Cells
6 DNA transcription factor SPIB and miRNA126 upregulated by natalizumab
7 JCV multiplies
CD8
8 JCV in peripheral circulation in B-Cells and as free virions
9 JCV enters brain and infects oligodendrocytes
10 PML initiates
a
b
c

Figure 2:



LEGENDS

Figure 1: Stages of PML pathogenesis

1. Initial infection through ingestion and/or inhalation of virion particles may lead to subacute infection and stimulation of antiviral antibody. No formal study has been conducted however to document these events.

2. In some individuals, approximately 30% globally, JCV infects the uroepithelium of the kidney and establishes a persistent or latent infection as evidenced by excretion into the urine with little if any pathological consequences.

3. JCV may escape into the peripheral circulation in some individuals and spread virions into lymphoid tissues including bone marrow establishing a latent infection that can be reactivated at times of immune suppression or modulation.

4. CD34+ cells in the bone marrow can become infected. Natalizumab forces consistent migration of CD 34+ cells to the peripheral circulation that continues for years during treatment.

5. Some of the migrated CD 34+ cells differentiate in a lymphocyte pathway, predominately in B cell lineage. Some of these cells that may be latently infected use these cells as host for viral multiplication.

6. Both DNA transcription factors like SpiB in the POU2A domain as well as miRNAs are temporally regulated by natalizumab and favor JCV multiplication in latently infected cells. Viral genome may undergo nucleotide rearrangement in the non-coding regulatory region from the urine associated archetype, less pathogenic form to the prototype, PML associated pathogenic form

7. JCV multiplication takes place in these cell phenotypes that may be recognized by CD4 and CD8 immune clearance as well as contributions of anti JCV antibody. Some infected cells escape immune clearance.

8. JCV can remain in circulating B cells, perhaps pre B cells, as well as non-cell associated, free virions in the circulation and traffic to the brain.

9. JCV can enter the brain in infected cells or free virus via hematogenous routes and initiate infection in the target oligodendrocyte. Mechanisms of viral entry are not well documented.

10. PML initiates as virus begins lytic, necrotic oligodendrocyte infection with gradual spreading of virus as evidenced by growing lesions in a multifocal pattern. a)represents MS lesions in PML patients treated with natalizumab; b)cortical white matter lesions with punctate lesions just below that are typical in PML and c)PML lesions in U fibers near the cortex.

Figure 2:

Natalizumab associated PML in an MS patient A, C, E: FLAIR images; B, D, F: Enhanced T1w images

Asymptomatic PML (A and B): Enhancing right frontal lesion with multiple smaller non enhancing punctate lesions (white arrow)

PML and IRIS (C and D): The lesion has enlarged on FLAIR and the enhancing area has increased; note the enhancing punctate lesions bilaterally (white arrows)

Post PML (E and F): Further enlargement of the lesion on FLAIR and presence of T1 hyperintense cortex (white arrow)

INSERT

Key Recommendations:

- Risk biomarkers for PML must be expanded and made more accurate
- Enhanced global data collection on cases of PML should be pursued to inform risk assessment and outcome analysis
- Recommendations for surveillance should be geared to risk profile
- Lower risk patients(<0.9 cases of PML/1000 exposed) should receive routine assessment for MS disease activity as part of disease modifying therapy selection and refinement as well as PML surveillance
- Higher risk patients (>0.9 cases of PML/1000 exposed) should undergo enhanced PML monitoring with more frequent MRI and antibody index assessments
- Updated risk assessments should be available as output from the surveillance network allowing best practices refinement of practice
- Patients with escalating risk factors should change therapy prior to PML detection

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A decade of lessons learned: PML pathogenesis and risks associated with therapies for MS

Introduction

Over one decade has passed since the first report of progressive multifocal leukoencephalopathy (PML) in several ~~MS~~ multiple sclerosis (MS) patients receiving natalizumab in a clinical trial. This monoclonal antibody to α4 integrins blocks inflammatory cell~~s~~ entry into the brain and blocked MS related clinical relapses. The occurrence of two very different demyelinating diseases in the brain of a single patient was unanticipated since PML and MS have very little in common except the destruction of myelin. ~~The etiology of~~ PML is a viral induced lytic brain infection while the ~~main~~ etiology of relapsing/remitting MS ~~remains an~~is an autoimmune response. PML in these MS patients was quickly associated with natalizumab, primarily because MS patients had been treated with other immune therapies for decades without reports of PML.[1-4] ~~The link between natalizumab treatment in MS patients and PML, however, was further confirmed as more cases of PML were identified.~~ Subsequently, t~~T~~here have been several ~~recent~~ rare reports of PML in MS patients on other therapies like dimethyl fumarates and fingolimod~~.~~ ~~but those numbers are very few.~~[5-9] The initial incidence of PML in natalizumab treated MS patients in the phase 3 trial was estimated to be 1 in 1000.[4] Ten years later, >7500~~0~~ PML cases have been reported with >20% fatality rate, and substantial morbidity to survivors. (Biogen, Tysabri Safety Update, September 2017, https://medinfo.biogen.com/secure/pmlresource) ~~and t~~The incidence in patients on ~~long term~~>24 months treatment~~,~~ ~~and~~ antibody evidence of JC Virus (JCV) exposure, and prior immunosuppressant treatment has reached at least 1 in 70, an incidence much higher than any other opportunistic infection in this setting.~~.~~[10,11] Use of a more quantitative antibody index has recently yielded estimates of 2.7% after 72 months natalizumab therapy with prior immunosuppressant exposure.[12] The incidence of PML in MS patients on other immune modulating therapies is much less, perhaps 1 in 10,000 to 1 in 100,000. So what have we learned from this experience on the pathogenesis of PML and how might that knowledge be applied to distinguishing therapy associated risks of PML that would help establish evidenced based monitoring of patients and inform the selection of effective MS treatments for individual patients? There

are several areas that surfaced from investigations on PML in MS patients that will be explored in this review: 1. Tthe molecular aspects of pathogenesis and cell specific involvement of JCV infection leading to PML, that are generally applicable to all PML cases regardless of various underlying diseases. ~~that may be generally applicable but also that specifically relate to unique physiological effects of natalizumab;~~ 2. the central role of magnetic resonance imaging (MRI) in the diagnosis of PML, monitoring treated patients to control its morbidity and advancing our understanding of aspects of its pathophysiology, and 3. new insights of clinical value of early PML detection. ~~These are three areas in which progress has been made on both PML and also MS disease pathogenesis.~~

Comment [c1]:

JC Virus infection and PML pathogenesis

To understand the complexities of the pathogenesis of PML, it is important to detail some of the background and biology of JCV infection leading to PML. First described in 1958, PML is usually characterized as a rare disease caused by JC Virus, named from the initials of the first patient from whom the virus was isolated in 1971.[13] PML develops in patients with compromised immune systems, particularly cell mediated immune responses. ~~However, there have been a small number of PML cases with no identified evidence of immune dysfunction.~~ Until the mid 1980s, PML was reported in patients with underlying neoplastic diseases, mostly lymphoproliferative diseases, and a few organ transplant patients treated with immune suppression for graft protection.[14] In the mid-1980s, HIV-1 infection became the predominate risk with up to 5% of AIDS deaths associated with PML. Effective antiretroviral therapy and earlier initiation to avert severe immunodeficiency have decreased the risk in HIV infected patients ~~with an approximate incidence of~~ to <1% .[15,16] Using a Pubmed search in 2017, we found that since 2005, reports of PML in patients with MS and other underlying diseases, and therapies to treat them, have increased 10 fold suggesting a greater awareness of PML based on clinical evaluation, MRI imaging and use of laboratory tests for JCV DNA and anti JCV antibody. It may be time now to consider PML not as just a rare disease but as a substantial neurological complication in certain high risk populations.

The pathophysiology of JCV in human hosts leading to PML is outlined in Figure 1 following steps 1 through 10 with additional details in Table 1, ~~and~~ further annotated~~ed~~ in Table 1. JCV has a narrow cellular host range and a variable effect on the organs it infects. Infection in human endothelial cells in the kidney[17-20] and in cells of hematopoietic lineage like CD34+, B cell phenotypes, CD19+ and CD20+ have little pathological effect making infection of hosts a silent event.[21] In the brain however, the multiplication in oligodendrocytes is lytic and results in PML with devastating clinical ~~consequencs~~consequences. Infection of the neurons in granular cell layer in the cerebellum can also result in a symptomatic neuronopathy. [22]

For cells to be susceptible to JCV infection, they need to express DNA binding proteins that recognize the viral genome, non-coding control region (NCCR) that initiates viral DNA replication and transcription for RNA and eventually protein synthesis (Figure 1steps 6,7/Table 1). There are a number of such transcription factors that are critical to JCV multiplication. [23] The noncoding control region (NCCR) nucleotide sequences are represented in two arrangements. The **archetype** NCCR is comprised of approximately 200 linear nucleotides in virions excreted in the urine, figure 1,step 2,which occurs in about 30% of the population. This 'archetype' variant is generally considered non-pathogenic in kidney or, if identified, in other compartments like plasma/serum and even in brain. Virus isolated from PML patient's brain, like the index patient JC, became known as the **prototype** variant associated with pathogenic PML brain and CSF.[23] These approximately 200 NCCR nucleotides are not arranged linearly but in direct tandem repeats of 98 nucleotide base pairs or other arrangements but always showing duplications. It is thought that the prototype variant is derived from the archetype by deletion and duplication. The tissue compartment or cell type in which a 'rearrangement' of the NCCR from archetype to prototype could take place is still not known but lymphoid cells are a very probable host. (Fig 1, steps 4,5).[24-27] While there are no specific studies in cell culture or in patients that show that such a rearrangement can take place, there are very compelling indirect data supporting such a mechanism. New evidence even implicates Epstein Barr virus coinfection as possible catalyst in the nucleotide transition of the archetype to the prototype variants.[28]

Sero-epidemiological studies show a global distribution of JCV with an estimated rate greater than 50% of the adult population having been exposed. [29]The initial site of infection is still not known but thought to be ingestion or perhaps respiratory inhalation. (Figure 1, step1,) Contact with JCV most commonly results in a subclinical infection during which individuals develop antibodies and cell mediated immune responses (Table 1).[30,31] The serology test used for these studies had been hemagglutination inhibition(HI) based on the virions' ability to aggregate human type O erythrocytes. Anti JCV antibody would inhibit that reaction. Using this assay, a recent epidemiological study showed a correlation of the percent of the population by decade of age, from 15% in juvenile ages to 80% in the 7th and 8th decade.[32] This observation has been verified with the development of ELISA assays that use recombinant produced VP-1 and not whole virions.[33] VP-1 is the major capsid protein making the outer structure of the viral icosahedral particle and functions in cell attachment. The commercial Quest Diagnostic assay, Stratify™ JCV and the next generation Stratify JCV™ Dx Select™ uses the same VP-1 produced by recombinant technology.[34] Based on these assays, approximately 55% of MS patients are JCV seropositive. There are patients who 'seroconvert' from negative to positive at a higher rate than those who 'serorevert'. The rates of seroconversion in either direction may range from 3% to as high as 10% over a course of years.[35] This observation can complicate testing for anti JCV antibody as part of a risk mitigation program for PML since presence of antibody indicates prior viral infection. An increase in antibody titer or index indicates a history of~~n~~ active infection resulting from a persistent or reactivation of latent infection. An algorithm using the antibody index, calculated using the optical density in the Stratify ELISA assay, shows a correlation with PML risk.[36] A substantial rise in antibody has been identified in PML patients both in their plasma and CSF for weeks or months after diagnosis.[37] There are also reports of an increase in antibody to JCV in natalizumab treated MS patients that is most likely due to release of a previous latent or persistent JCV infection with a subsequent antibody response. The value of regular intervals of anti JCV antibody monitoring is important but should be put into context. The antibody results indicate whether a patient has been exposed and if titers/index increases substantially may indicate that an active infection has taken place. However, reliance on antibody titers/index change is limited as illustrated by the apparent absence of increased titers in patients with prior immunosuppression. However,

fundamental virology principles across most viruses that can become latent accept that individuals who have been exposed to a virus are at greater risk for disease from that virus than those who have not been.

In addition, anti JCV antibodies may be directed to different regions of the primary capsid protein, VP-1, that could be unique to a particular patient. As is the situation with the viral NCCR, the VP-1 gene can be hypervariable producing a number of VP-1 proteins with different primary amino acid sequences compared with the prototype variant. This has been known for many years in the thorough description of the 'JCV Type' linking geographical locations with independent V-P -1 genes and protein variants.[38] So it is not surprising that any one PML patient may have multiple representations of VP-1 protein at any one time. This observation had led to the hypothesis of VP-1 gene rearrangement that could result in a more 'neurovirulent' variant leading to PML.[39,40] This observation warrants further investigation that would require 'deep sequencing' studies.[41] However, it appears that PML patients are infected with the prototype VP-1 protein since that is the antigen used in the ELISA assays of commercial, academic and government laboratories. It is possible that immune escape of JCV VP-1 variants could occur due to either persistent JCV in cell compartments or mutations in the VP-1 gene to avoid immune recognition.[40] This has been an area of new investigation in the last several years.

~~Although~~ However, antibodies~~y~~ to JCV may not ~~likely~~ result in protection against PML development (and are thus not necessarily neutralizing antibodies)~~.~~, There is ample in vitro data showing antibody made against JCV blocks virion adsorption to target cells that limits attachment and entry, thus reducing viral multiplication. But there is little clinical evidence in healthy people or patients showing antibody may help control JCV infection.[42] In fact nearly all individuals who persistently shed JCV in their urine are seropositive. Some seropositive individuals can even be viremic, and PML patients can have very high levels of CSF antibody in the presence of high viral DNA copy numbers.[43,44] Consequently, CD-4+ and CD8+ cytotoxic cell recognition of viral antigens probably play a ~~pivotal~~ more significant role against JCV infection. CD8+ cytotoxic T cells to JCV prototype VP-1 have been identified in PML and non-PML patients for many years.[45,46]~~,~~ (Fig 1,step7) With the increase reported incidence of PML in MS patients treated with DMTs, newer studies have identified CD 4+ T cells as critical to control of JCV in natalizumab treated patients directed against the 4

major JCV proteins, T antigen, VP-1, VP-2, and agno. In addition, lack of CD 4+ cells and those releasing Il-10 were identified in natalizumab treated MS patients including one of the index cases who remained persistently JCV positive in CSF for years.[47] CD 4+ T cells have also been cultured from brain tissue of PML patients that are directed to potential more neurotropic viral capsid proteins not identified by CD 4+ T cells in the periphery. These CD 4+ cells seemed necessary to stimulate cytotoxic CD8+ cells to function for clearance of JCV from the brain so perhaps were lacking in PML patients. These laboratory and clinical studies have been undertaken directly in response to the need for a better understanding of the immune system role because of PML incidence in MS patients. Perhaps in further defining risk factors for PML, identifying CD 4+, CD8+ and other immune system cells for activity to JCV antigens would be informative in the PML high risk patients.

Unlike MS, the specific etiologic cause of PML is well known, JC Virus lytic infection of oligodendrocytes. To acquire a deeper understanding of the pathogenesis of PML, there needs to be a magnified focus on the virus including the stages of infection leading to oligodendrocyte cell death. (Figure 1, step 10)

A case can be made that common pathophysiologic pathways explain the steps leading to PML, regardless of the underlying risk that allowed it. For example, some patients with T cell immune compromised systems may harbor JCV in a latent state in tissues like kidney, lymphoid organs like bone marrow, and possibly brain. Periodic JCV release from latency or even a persistent infection is poorly managed by the immune system so virus may enter the brain as free virions or through an infected cell (Figure 1, step 9/Table 1). CD 4+ cells that do not adequately recognize JCV antigens have now become an important part of lack of immune surveillance[48] while cells in the B cell lineage have been implicated as possible carriers since JCV has been identified in CD 19 and CD 20 cells.[49] The brain is not the initial site of JCV infection and data on latency in brain are very limited. ~~and not described in MS patients.~~ There have been reports of identification of JCV DNA in brain tissues of non-PML patients.[50] In these reports, there was no evidence that the entire viral genome was present in order to initiate and sustain viral multiplication. ~~In fact t~~There is only one study that specifically investigated the presence of JCV DNA in MS brain tissue and ~~did not find any evidence~~found it absent.[51] A multicenter study using

blinded samples and controls of positive and negative brain tissues should be considered to determine the existence of latent JCV in the brain. However, at this point it is more ~~It is~~ likely that release of latent JCV in the periphery, particularly the virulent variant, is a key factor. The kidney/urine derived variant is considered non-virulent or at least less neurotropic, so the best candidate for latency is likely in lymphoid cells (Fig 1, step 4,5,Table 1). [52]These cells can be hosts for rearrangement of the viral NCCR and perhaps gene rearrangement of the VP-1. They would be subject to factors that activate viruses like EBV that may even assist in JCV NCCR rearrangement from the archetype to the prototype by gene rearrangement and insertion as well as the potential to be targets for RAG 1 and RAG2 enzymatic mechanisms best known for their role in immunogloblulin diversity.[53,54]

So a question becomes what unique features does natalizumab possess that no other therapy associated PML risk shares. Natalizumab associated PML patients are not systemically immune suppressed. Other opportunistic infections are not prominent, suggesting PML is a specifically enhanced problem rather than the result of broad immunosuppression. Further, it appears to require years for the risk to be manifest. These two factors highlight the need to understand PML pathogenesis beyond pure immune suppressive explanations. It may be over simplistic to suggest that lack of immune surveillance is the major underlying mechanism of PML in natalizumab treated MS patients. Even with immune reconstitution inflammatory syndrome (IRIS), some natalizumab treated PML patients continue to have detectable virus in CSF for months to years.[37] We see two unique features of natalizumab that contribute to its special risk. One is that natalizumab forces migration of hematopioietic stem cells, CD34+ and precursors of B cells from the bone marrow (Figure 1, step 4). It shares this feature with efalizumab, the other monoclonal with highest risk of PML. The other is the temporal relationship of PML incidence after long term dosing, approximately 2 years or longer. JCV can be latent/persistent in CD 34+ or preB cells in the bone marrow described by several laboratories[24,25,55] and in culture models identifying DNA binding factors that act on the JCV transcription sites.[56] These factors can also be found in CD 19 and CD 20 cells in the peripheral circulation. It is possible that the high percent of such cells forced out of the bone marrow for long periods would result in release of some latently infected cells (Figure 1, step 5). In those individuals, perhaps their immune system cells do not completely clear newly released virions particularly if remaining intracellular like EBV. But that observation does not account for the temporal correlation of the high

incidence of PML after nearly 2 years of dosing. However, natalizumab also upregulates genes in a critical pathway for maturation of B cells, POU domain DNA transcription factors particularly Spi B that binds JCV NCCR. The time course of natalizumab effect on POU domain regulation is consistent with PML incidence. [57,58] The two characteristics only occurring in natalizumab, forced migration of cells from the bone marrow and temporal upregulation of factors that highly favor JCV growth match the current observations of delayed PML incidence and focuses attention on the cause of PML, JC Virus cellular interactions leading to PML (Table 1, steps 5,6). While perhaps still premature, it is noteworthy to consider how laboratory analysis of these factors in immune cells as well as immune cell antiviral function would further identify PML high risk patients before oligodendrocyte infection is initiated.[59 60]

MRI Imaging for Early PML Detection/Diagnosis/Management

The approach to diagnosis of PML has been reviewed elsewhere, but routinely requires identification of active CNS pathology and JC virus in the brain.[61] Brain imaging is a critical contributor to the diagnosis of PML.[61] Indeed, without an MRI lesion, PML diagnosis cannot be verified. The sensitivity of MRI in identifying PML lesions has made it the modality of choice in monitoring natalizumab treated MS patients for early detection of PML. Consideration of imaging in relation to the clinical stages of PML requires understanding the clinical manifestations that PML takes, depending on the degree of brain infection, as well as the status of immune response to this unique infection. (Table 2) We define onset of PML as the time JC virus enters brain and infects~~active brain infection of~~ oligodendrocytes~~ by JCV~~, which ~~predictably~~ ultimately leads to a clinical serious brain injury. Table 2 emphasizes that there is a pre-symptomatic period during which the infection grows which even b~~m~~y MRI is likely to be 3-6 months in duration.[62] This accounts for some of the low risk of early months of ~~risk~~therapy, as well as the interval when PML is most likely to be seen after stopping natalizumab and transitioning to a low risk therapy. The symptomatic disease state is very different depending on whether immune reconstitution is achieved or not. ~~The~~Without ~~therapy~~immune reconstitution, the "classic" PML~~disease~~ is generally fatal, and

no effective immune response is generated. ~~With~~ Alternatively, as generally occurs in natalizumab cases, successful immune reconstitution ~~(generally guided by therapy, but occasionally spontaneous), an i~~precipitates an inflammatory ~~change occurs in PML lesions~~syndrome that can arrest the disease. This response must come quickly enough to avert death from disease progression, but when it occurs and the patient survives >6 months, the ~~clinical~~ viral disease is generally controlled, albeit with a fixed brain lesion seen in post-PML survivors. (Table 2) PML therapy has been reviewed in detail elsewhere.[63] No anti-viral therapies, including widely used mirtazapine and mefloquine[64], have been demonstrated to improve outcomes, but it is abundantly clear that immune reconstitution changes the course of PML for the better. The concept of using plasma exchange to hasten immune reconstitution with natalizumab cases is thus a rational approach that has been widely adopted and associated with PML outcomes that outpace historical precedents.[65] However, the balance of concerns about potential augmentation of damaging IRIS remains a concern that clinicians must balance.[66,67] Similarly, active use of corticosteroids or maraviroc[68] to blunt IRIS remain controversial, but at least in more advanced disease active immune reconstitution seems likely to contribute to better outcomes. Gathering informative data to more clearly articulate recommendations remains extremely challenging with this rare and serious disease. Urgency for early diagnosis of PML (Table 2, 3), preferably before the onset of clinical symptoms, aims at limiting brain damage and thus disability. Recommended MR parameters are widely available. Increasingly, annual scans including brain and spinal cord are recommended to monitor the efficacy of DMT for MS (Table 3). Even more frequent scans of brain alone are recommended seeking early detection of PML in higher risk settings. Retrospective analysis of some PML patients with frequent scans demonstrates lesions developing months before symptoms.[62] It is now recognized that development of PML symptoms may only occur months after JCV enters the brain and forms a visible lesion with MRI. ~~Reversal of enabling factors (like natalizumab) before any symptoms develop is thought to limit brain injury.~~ To date, we found 19 publications [2,62,69-83] reporting on 48 PML patients asymptomatic at the time of a detectable lesion. Twenty-one of these patients developed symptoms in up to 41 weeks after lesion visualization and in a further 13 patients natalizumab was withdrawn before the development of symptoms, with 4 patients remaining symptom free. Disabling outcomes including mortality appear to be reduced in these patients.[82]

It is critical to be aware that verified PML lesions actively evolve on repeated imaging, either because the JCV induced disease progresses, or because the inflammatory response controlling the infection results in evolution of the image characteristics. Thus, repeated MRI images that do not change help rule out PML, while evolving lesions are consistent with a PML diagnosis. PML may not be diagnosed on a single MRI without additional clinical and virological confirmation.

Despite the increasing number of PML cases reported, the low frequency, sporadic appearance, and uncontrolled clinical market status of natalizumab distribution, make a prospective assessment of the sensitivity, specificity and accuracy of imaging difficult. It has been suggested that the 4 most helpful features suggesting a PML lesion (applicable to lesions in asymptomatic patients) are its subcortical location (involvement of U-fibers), T1 hypointensity, DW hyperintensity, and the presence of punctate T2-hyperintense lesions.[80,84] (Figure 2) Unlike AIDS associated PML, GDd~~(Gd)~~ contrast enhancement is often seen even at presentation in PML in the setting of treated MS. Occasional cortical and deep GM involvement can occur but white matter distribution dominates PML.

The punctate lesions may offer some insight into the pathophysiology of PML, suggesting an inflammatory response in the lesion. Recognition of this imaging pattern has emerged in settings where partial immune response to JC virus is commonly present, and was not noted in the era when most cases were AIDS associated and lacked inflammatory response on pathologic exam. Punctate lesions appear to develop in perivascular spaces in the brain, where JCV in mononuclear cells and infected glial cells has been identified.[25] Histological examination has shown that inflammation typical of ~~immune reconstitution inflammatory syndrome (~~IRIS~~)~~ to JC virus is associated with a marked infiltration of CD8+ T lymphocytes, especially in the perivascular spaces.[85] The frequent observation of this pattern thus may be a marker of IRIS, and is consistent with the early evidence of contrast enhancement suggesting IRIS in many natalizumab associated cases of PML. While punctate lesions often enhance with GD, their unenhanced presence on T1suggests pathology outside intrinsic brain cells supporting JCV replication, and thus may instead specifically reflect~~s~~ inflammatory response. The alternative interpretation that these are the smallest “islands” of demyelination in early infection is plausible, but their early enhancement favors their location in relation to blood vessels with increased permeability to GDd. If these lesions reliably

represent disease with IRIS, they could direct clinicians to focus on anti-inflammatory therapy for these patients. Another interesting MRI lesion similarly reflecting probable inflammatory responses is a T1 bright subcortical lesion that is often associated with seizures and inflammatory PML lesions.[86](Figure 2)

Confirming the Diagnosis of ~~Asymptomatic~~ Pre-symptomatic PML

The success of frequent MRI brain imaging will be measured by the identification of increased proportion of asymptomatic lesions determined to be PML. AAN Diagnostic Criteria[61] require symptoms for definite diagnosis, yet ideally PML would be detected and arrested without symptomatic brain damage occurring through close MRI monitoring of high risk patients. Verification of a PML diagnosis without symptoms is challenging. Very early, CSF viral load may be low or undetectable and the dynamic nature of PML cannot be confirmed by a single scan. ~~Nonspecific white matter lesions are common, and in MS patients lesions may be part of the underlying disease. While~~ MRI lesions may be characteristic of PML, but no MRI features have been described as being pathognomonic. Small lesions can be difficult to differentiate from MS lesions especially when there is a high lesion load.[83]

A critical clinical point is that in patients at risk, new MRI lesions consistent with PML should be assumed to be PML, and active longitudinal diagnostic and therapeutic steps including repeated CSF sampling (if required), repeated MRI imaging, and serial JCV antibody titers should be performed to help establish the diagnosis. During these procedures clinical management should be pursued as if PML is present. Delay in managing PML by awaiting AAN definite diagnostic criteria, would sacrifice the benefits of early detection gained by monitoring with MRI. Such an approach was successfully implemented in at least 3 patients with PML compatible MRI changes but negative CSF JCV PCR.[69,71] In these 3 patients managed as if the diagnosis was established, two had subsequent detection of JCV in CSF on repeat sampling. In all patients the MR imaging evolved to a pattern compatible with development of PML with IRIS, helping to strongly support the diagnosis. Often asymptomatic patients later develop symptoms associated with IRIS, ultimately fulfilling traditional diagnostic criteria.

To date, serial quantitative JCV antibody determinations have too rarely been used to help consideration of possible PML in

difficult cases. Active JC virus disease including PML typically drives an increase in JCV antibody titers that confirms JCV related disease. Thus, even if viral DNA is not demonstrated in CSF, if compatible and evolving MRI lesions are associated with increasing systemic JCV antibody titers, this should provide significant support for diagnosis of PML.[87] Use of this approach may not work in the face of prior immunotherapy, however, necessitating biopsy or presumptive diagnosis without confirmation. Brain biopsy remains the ultimate criteria when a definite diagnosis is required lacking detection of viral DNA in the CSF. However, with small pre-clinical lesions, it will be difficult to biopsy at the earliest stages, and should be only used judiciously when certainty about the diagnosis is clinically critical.

Risk Mitigation Strategies Are Failing Us

A risk mitigation strategy was developed by Biogen/Idec ~~following FDA requirements~~ to protect patients from developing PML in the setting of natalizumab therapy.[88] The fundamentals have been actively discussed, and variably applied. [10,11,89-93] However, the ideal of witnessing plummeting incidence of PML cases has not yet materialized. [94] We summarize our own ~~detailed~~ suggestions based on a most recent ~~suggestion~~algorithm [95] ~~(101 McGuigan)~~ and the data we are aware of in Table 3. We propose the surveillance be guided by the estimated risk, dichotomizing it into 2 groups: a) regular surveillance if the PML risk is $\leq$ 0.9/1000 and b) intensive surveillance if it is above 0.9/1000 patients. This approach allows simple adjustments when the estimated risks change or new risks are identified. (Insert: Recommendations)

Shortcomings of risk stratification elements

The substance of the three key risk stratification elements is known to have flaws that might help understand the suboptimal impact they exert. First, while JCV antibody is a predictor substantiating infection with the virus causing the disease., ~~its imperfections increase with careful inspection.~~ Unfortunately, JCV viremia and viruria can be present in antibody negative patients.[96] Further, quantitative antibody analysis, while suggestive of more active infection with higher risk, fails to be predictive after prior

immunotherapy. While overall expression of antibodies inversely correlates with disease risk, some evidence that antibodies still may play a role in controlling this virus is emerging, reviving interest in vaccination strategies for JC virus or PML management.[42,97] Thus, JCV antibody status falls far short of an ideal biomarker. Second, duration of therapy as a risk parameter is also flawed. The measured variable is duration of time ~~from~~ from ~~DMT~~ natalizumab therapy start to clinical diagnosis of PML, which itself may be a considerable time after the first symptoms.[98] The actual biologic interval of interest is time to brain infection with the virus. Through observations with more intense monitoring of pre-symptomatic high risk populations, we now realize that infection likely takes place at least 6 months prior to the clinical manifestations of disease, substantiated by observation of pre-~~a~~symptomatic lesions of PML on MRI scans, and by pre-symptomatic immunoglobulin elevations leading up to PML diagnosis.[29,82] It is likely that the pre-symptomatic interval is even more variable related to the eloquence of clinical expression of lesions in different brain regions. For example, it seems ~~given~~ likely that brainstem lesions would more rapidly lead to symptoms compared with frontal lobe lesions. Thus, extrapolation about specifics of pathophysiology based on the crude interval from start of therapy to clinically symptomatic disease is quite imprecise. Recent critical analysis about the imprecision of the Biogen risk estimates for impact of duration of infection become even less meaningful, when the imprecision of biology reflected by the measure is considered more critically.[92] Third and finally, the impact of prior immune suppression on risk is similarly quite poorly fleshed out in literature. It is fundamentally untenable that the specifics of type and duration of prior immunotherapy is of little consequence in determining risk on a biologic basis, yet this is at present a monolithic consideration. A dose of azathioprine would receive equal weight with long term cyclophosphamide therapy, yet impact on the immune system must be very different.

Thus, current negative commentary on the precision of the present risk mitigation strategies is unsurprising, but perhaps clinically not so critical.[10,92,99,100]

~~*Considerations from the clinical decision process*~~

~~While providing plausible sources of additional PML risk, none of the established factors truly drives appropriate clinical decisions by patients and their physicians about when they should use a drug with a recognized risk of PML. Consider the decision of a patient with aggressive relapsing remitting MS who is experiencing increasing neurologic disability. If a therapy has the possibility to halt disease progression in a majority of cases, but there is a slight moderate risk of death (~25% of the small number of PML cases) or instead, the risks of therapy may sound reasonable to an individual with a risk affirmative approach to living. At present, weighing this decision is left to the patient and physician.[94] The problem is really how to ethically relay the needed information so a patient can either accept the risk openly or reject it. {Kramer, 2017 #15776}Better ways to assure a balanced discussion are required as well as assuring that clinicians are enabled and reimbursed to spend the time necessary for this nuanced discussion. Therapeutic enthusiasts must not minimize the serious risks of therapy with PML risks, while timid clinicians should not fear offering this choice.~~

Comment [c2]: this is not one of the strongest sections, and since we are asked to try to shorten the overall manusript, and it is a very superficial pass at these issues, I suggest we cut this...

Risk stratification with newer disease modifying MS therapies

~~Even the~~The ~~crude~~ risk mitigation developed for natalizumab is likely only truly applicable in relation to that drug. PML risk with other available and emerging DMT for MS (dimethyl fumarate, fingolimod, rituximab, ocrelizumab) is much lower,[89] and while its presence must be acknowledged, it should not severely impact decision making where benefits can be accrued by implementing early and effective MS therapy. In the case of dimethyl fumarate, monitoring for lymphopenia appears likely to identify a higher risk group in whom alternate therapy should be sought. In that setting prolonged lymphopenia with absolute lymphocyte counts <750 accounts for most cases, although the risk may reside particularly in the loss of CD8 cells critical to JCV control.[101] Measurement of circulating lymphopenia however is not universally helpful. For fingolimod, this strategy cannot be applied since circulating lymphocytes decline while effective lymphocytic function appears largely normal. Similarly, alemtuzumab associated risk for PML has not been demonstrated in MS patients yet despite marked impact on lymphocyte profiles. Alternatives to lymphocyte counts might include serial antibody measurements, or monitoring for circulating JC virus.~~,~~ The multiplex PCR that allows identification of

emergence of prototypic virus that likely has enhanced risk of PML seems a plausible means of risk stratification, but has not yet been demonstrated to serve in this way.[23] However, the low overall risk with alternative DMT makes it difficult to validate, and probably impractical to use as a stratification factor in practice. Other alternative PML risk stratification approaches under investigation in natalizumab associated PML include measurements of CD62L and lipid-specific IgM bands. [102,103]At present similar logic applies to rituximab and ocrelizumab. These monoclonal antibodies directed against B cells have yet to demonstrate excess risk of PML in MS patients despite a large number of cases associated with rituximab when used in the setting of hematologic malignancies and other diseases with greater underlying risk of PML.[104,105] The theoretical risk suggests clinical vigilance, but no other risk mitigating strategy can be recommended for PML at this time when using these emerging MS therapies.

Thoughts on risk mediation based on what has been learned

Ongoing consideration of additional risk mitigating factors that would aid risk assessment and be more predictive should be a theme of investigation-. Technology that allows more detailed consideration of JCV specific immune control might more accurately reflect risk. Quantitative definition of the specifics of T cell recognition and response, as well as identification of the emergence of prototypic virus might well alert the clinician to a small subset of high risk patients in whom therapy would be foolhardy.

The fact that the present system has largely deluded us all is evidenced by the lack of impact so far on the incidence of new cases. While the imprecision of the present risk model is likely in part to blame, the most likely cause is that risk monitoring and communication is either too inconsistently done to inform patients, or that they are choosing to continue to use natalizumab even when they have a significantly elevated risk. ~~The overall good from added quality of life in successfully treated patients may well exceed the real harm to the smaller number of patients who unfortunately develop treatment related PML. To assure that our system is offering better outcomes overall, and that people are not being treated without proper understanding, we need better longitudinal understanding of both the risks and benefits of our therapies.~~

Development of therapeutics for auto-immune diseases including MS, genetic origin of immune disorders or neoplastic disease is in evolution, and optimizing these choices to include PML risk will require more detailed data than currently exists. For example, the relative efficacy of MS therapies, as well as their costs must inform prescribing patterns. Estimates of these factors are difficult to substantiate. These factors must be integrated with the risk of PML encumbered by various therapies. ~~At present, natalizumab is known to have substantial risk, while there is evidence that both fingolimod and dimethyl fumarate elevate PML risk in MS patients slightly.~~[7-9,106-108] ~~Integration of these risks with relative tolerability and efficacy of the agents invites refinement of clinical skills of physicians, and challenges them to find ways to reduce the risks for all patients requiring any of these therapies.~~[106] Calculation of all of these factors, and explaining them to a patient who must fit this evidence into a personal risk tolerance profile is a very difficult task. Better tools need to be developed to assist patients and physicians in meaningful ways to understand this and come to an ethically sound decision for the patient's management.[106]

Conclusion and future directions

The past decade has witnessed substantial progress in understanding JC virus and PML. The close observation and additional cases seen in multiple sclerosis patients has given the opportunity to enrich the molecular biology of JC virus, and to make some progress on likely evolution of risk and invasion of the brain. Enhanced identification of higher risk patients has allowed the evolution of use of MRI, such that detection of PML lesions prior to symptom onset is commonplace in high risk patients. Improved use and interpretation of MRI have proved pivotal for PML. However, the clinical management of MS patients remains challenging.

Further, the outcomes from PML have markedly improved. While PML is still a serious and sometimes lethal disease, a majority of patients contracting it survive in settings where immune reconstitution is possible, and with early detection of disease, commonly

severe disability from PML can be avoided. However, we still are unable to detect individual risk precisely enough to give easy instructions about PML, and still settle for very early diagnosis to minimize injury.

Meanwhile, the practical means to enhance communication about risk and help patients select the optimal approach to their illness tailored by their own willingness to take risk is an ongoing clinical challenge. It is especially important to be sure that it is not for lack of monitoring and acceptance of known risk that patients are developing PML. If on the other hand, patients have accepted the risks and continued therapy with full knowledge of risks and benefits, principles of ethical care have been served. Ultimately understanding the overall difference in outcomes of those who accept the risk with DMT and do well, compared to those who develop PML, should be understood and have acceptable value, if the choice to use this therapy is to continue to be up to patients and their clinicians.

The basis for such an analysis is the availability of credible data. PML is not a reportable disease, and detailed retrospective data gathering is laborious and incomplete. Registration of cases with systematic reporting of circumstances of the disease would allow us to study the impact of risk mitigation concepts. Development of widespread or universal data collection and consideration of cases could speed research on risk and outcomes, and allow more precise risk mitigation programs. We believe that while the mitigation strategies are not perfect, the largest failure is in not implementing changes in therapy when risk is known to be ~~increase~~elevated.~~ It will be easier to implement changes in therapy when~~ With MS therapies that are comparably effective to natalizumab, we believe replacement of natalizumab in high risk patients should be more uniformly employed and should reduce the burden of this tragic disease. ~~but safer alternatives are available to substitute for natalizumab.~~

Table 1 – Stages of JCV Infection Leading to PML

TABLE 1:	Urine[a]		Blood[b]		CSF[c]	
	Antibody[d]	DNA[e]	Antibody	DNA	Antibody	DNA
A. Primary Infection[30, 31] (Ingestion or primary inhalation)	Not measured	Not symptomatic so rarely measured	Emerging in juvenile years (15%) increasing to 80% in 7th and 8th decade) Seroconversion 3-10%	Not measured	Not measured	Not measured
B. Establish Latency in kidney [17-20]	Not measured	Can be undetected to low or >$10^{6/7}$c/ml; sporadic or continual release	Variable levels from low to high titers	Can be transiently detected generally <10^2 c/ml	Not present	Not present
C. Escape from kidney to circulation; may enter lymphoid organs like bone marrow[21,24,26,27,53,56]	Not measured	Can be undetected to low or >$10^{6/7}$c/ml; sporadic or continual release	Variable levels, % population seropositive increases with age	Can be transiently detected generally <10^2 c/ml	Not present	Not present
D. Escape into circulation, in cells or free virions enters brain, infects oligodendrocytes[28,32,48,49,57]	Not measured	Can be undetected to low or >$10^{6/7}$c/ml;	Detected at variable levels, titer increase during onset of PML	Transiently detectable, variable levels commonly 50 to > 500 c/ml	High titer or index indicating intrathecal antibody production	Generally detectable at 10-10^7c/ml

Footnotes for Table 1

a. ***Urine samples*** can be tested for JCV DNA to demonstrate a latent or persistent infection. Approximately 30% or more of the population globally excrete JC Virus in the urine, viruria, without pathological effects in the kidney although very high levels of viral DNA can be present. The JCV ***archetype*** variant, unique arrangement of non-repeating nucleotide sequences in the non-coding control region or NCCR, is the most common in the urine. The JCV archetype variant is thought not to be neurotropic and is rarely detected in brain or CSF of PML patients.

b.Blood samples are tested for antibody to JCV in either serum or plasma indicating prior exposure to JCV. Seroepidemiology of JCV has shown a global presence of JC Virus infection. However, multiple alterations throughout the genome can be found that have used to 'Type' JCV allowing studies to define geographical distribution and follow transmission in family members. High levels of antibody or increasing levels of antibody, reported as a titer or index, usually indicate active infection from reactivation of latency or a new infectious episode. Antibody levels also may fluctuate so sero-positive or sero-negative status may change over time. Estimates that this conversion takes place in 2% to greater than 10% of the population annually. Also, rare seronegative individuals may experience JCV infection and not show or make antibody as evidenced by viruia or viremia.[43]

Blood samples are also used to test cell compartments for JCV DNA as cell carriers for infection or persistence. Notably viral DNA has been found in CD19+/20+, CD34+ cells but not in CD 3 (T cells) or monocytes. [21]

c. ***CSF samples*** with detectable JCV DNA serve as the laboratory confirmation of PML diagnosis. Quantification of viral DNA is reported as genome copies per ml. Currently, the most sensitive assay has a limit of detection of 10 c/ml.[23] Usually, the lower the copy number of viral DNA the better the PML prognosis. Viral DNA in the CSF is the ***prototype*** variant with repeat nucleotide sequences in the NCCR thought to be derived from the ***archetype*** variant through deletion, duplication and rearrangement. This transformation from the archetype to prototype probably takes place before entry into the brain in lymphoid tissues like nodes or bone marrow.[52,53] There can be multiple nucleotide arrangements of the NCCR in PML patients plasma, brain and CSF. However, generally no two PML patients demonstrate identical patterns although the same variant is found throughout an individual PML patient's tissues. [41]

CSF samples also can be tested for intrathecal antibody to JCV that occurs in PML patients and may be used as a marker or sign of developing PML.[43]

d***. Antibody*** is measured by ELISA assay using viral major capsid protein, VP1 derived from the prototype variant, as antigen; result reported as titer [33]or index[34], depending on assay, reflecting the level of antibody response, usually IgG. Other VP1 variants have been reported that may have escaped immune control with mutations in the VP1 sequences that were identified in the CSF of PML patients with specific T cell responses so attributed as the potential PML causative variant.[40] However, almost all PML patients with ELISA demonstrated antibody to JCV have the prototype variant in brain and CSF.

e. Viral DNA is measured using quantitative polymerase chain reaction specifically measuring copy number of viral genome DNA as c/ml; low=50c/ml; hi=>500c/ml. In MS/PML patients, the median c/ml is >100c/ml to <500 c/ml in a range of 10c/ml to 10^7c/ml [37] Primary infection is subacute, can occur in infancy from parental transfer to early juvenile years as determined by antibody presence.

Table 2: Stages of Progressive Multifocal Leukoencephalopathy

	Pathology	Duration	Blood		CSF		MRI
			Antibody	DNA	Antibody	DNA	
A. Pre-Asymptomatic PML	Unknown, likely as in "Classic" below	3-6 months estimated from viral entry to brain until neurologic sx[1]	Increases, dynamic increase supports PML dx	Transient, 50 to 500 c/ml	Detectable, titer increasing	Generally low titer detectable, 10-10^7 c/ml	New Lesion on surveillance MRI, generally small, DWI bright
B1. "Classical" Symptomatic PML without immune responses[3]	Demyelinating plaques, bizarre astrocytes, oligodendrocytes with nuclear inclusions, notably absent inflammatory response	3-6 months from onset of sx to death ~~, leading to death~~ if no immune reconstitution	Increases, marked increases typical of PML	Transient, 50 to 500 c/ml	Detectable titer, increasing	10-10^7 c/ml, rarely undetectable	Typical brain lesions[2], enlarging, rare if any CE[4], no mass effect
B.2 PML with immune reconstitution inflammatory syndrome (IRIS) [4]	Classic pattern plus inflammatory response with variable mix of CD8 and CD4 lymphocytes, may have declining amount of JC virus	~~12~~-5 months after immune reconstitution[5], associated with potential for survival of PML, may be present at diagnosis in natalizumab PML setting	Increases	Transient, 50 to 500 c/ml	High titer	10-10^7 c/ml, may rise then fall during course	Typical brain lesions[2], CE usual but not required. [4] Punctate pattern and T1 bright cortical line suggest this stage of PML.
C. Post PML survivors[6]	Atrophy, fibrosis, rare JCV infected cells	Years, dependent on underlying disease(s), fixed lesion may support clinical	Little data, but likely relatively stable at high titers	Transient, 50 to 500 c/ml	High Titer	Often undetectable, but may remain detectable[7]	No CE, Brain atrophy in region of prior lesions, Defect with ↑

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		improvement 6-12 mo after dx, then is stable clinically in most cases					T1 cortex

[1] Duration of pre-asymptomatic stage dependent on location in brain. Clinically silent regions like frontal lobes may harbor pre-asymptomatic infection for up to 41 wks in one documented case, while areas causing symptoms with small lesions will be detected in fewer months. Duration of this phase is consistent with symptomatic PML occurring up in the first 6 months after natalizumab discontinuation.

[2]Four most helpful features suggesting a PML lesion in asymptomatic patients: subcortical location (involvement of U-fibers), T1 hypointensity, DW hyperintensity, and the presence of punctate T2-hyperintense lesions. Evolution of lesion on subsequent scans important to substantiating diagnosis.

[3] Typical of PML developing in untreated HIV/AIDS or highly immune deficient setting where virutally no immune response seen. Classically this symptomatic disease led to death within 6 months in most patients.

[4] PML with IRIS can also occur at the pre-asymptomatic and symptomatic phase where partial immune deficiency occurs (common in natalizumab MS at onset); punctate lesions (± CE) and T1 cortical bands probably indicate inflammation with or without CE. Prior cortiocsteroids reduce chance of CE without eliminating inflammatory response. CE: Contrast enhancement

[5] PML with IRIS persists for up to 5 months or longer documented by late biopsy of lesions, and may require repeated therapy to suppress.

[6] Deaths from PML typically occur within 6 months of diagnosis, with survivors dying of other causes months to many years later, often hastened by underlying diseases, or the hazard of neurological disability and its complications.

[7] JCV DNA typically declines and often is undetectable after survival of PML. However, virologic cure does not occur, and CSF may continue to have generally low level detectable virus indefinitely.

Table 3: Protocol for PML surveillance in MS patients

Monitoring steps for Patients							MRI Sequences						
Treatment Group	PML risk		S	Anti JCV AB	MRI		Brain					Spine[1]	
				Frequency	Frequency	Indication							
	Treatment Duration	Risk Estimate /1000[a]					FLAIR	T2	DWI	T1	T1+Gd	PD/T2/STIR	T1+Gd
Immunmodulatory treatment					Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
All NTZ patients					Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Anti JCV negative	NA	NA		6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Anti JCV positive, no prior immunosuppression													
Index <0.9	1 – 72M	0.1 – 0.6	**R**	6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
Index 0.9-1.5	1 – 36M	0.1 - 0.8	**R**	6M	Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	37 – 72M	*2 - 3*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-
Index >1.5	1 – 24M	0.2 – 0.9	**R**		Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	25 – 72M	*3 - 10*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-
JCV +ve, Previous Immuno-suppression	1-24M	0.3 - 0.4	**R**		Yearly	MS activity	Y[2;3]		(Y)[4]	Y[5]	Y	Y[8]	Y[9]
	25 – 72M	*4 – 8*	**I**		3-4 M	PML	Y[6]	Y[7]	Y	-	-	-	-

Abbreviations: FLAIR = fluid-attenuated inversion ~~recovery ,~~recovery, DWI = diffusion weighted imaging, I = intensive surveillance, S = surveillance, T1 T1 + Gadolinium, PD = proton density , STIR = Short T1 inversion recovery; NTZ= Natalizumab, R = regular surveillance

[a] Risk estimates from report of EMA, February 2016[107]

MS: Monitor MS activity, justified by aim to provide DMT achieving "no evidence of active disease" (NEAD) for optimal clinical management of MS

PML: PML Surveillance

[1]Spine MRI is not indicated for PML monitoring, but may be used in monitoring MS disease activity, or if neurological exam suggests possible spinal cord localization of pathology

[2]Rovira[108]: **Mandatory** a) Axial proton-density and/or T2-FLAIR/T2-w; b) 2D or 3D contrast-enhanced T1-w

Optional a) Unenhanced 2D or high-resolution isotropic 3D T1w; b) 2D and/or 3D dual inversion recovery; c) Axial DWI

[3]Traboulsee[109]: **Mandatory** (Core) a) Anatomic 3D inversion recovery–prepared T1 gradient echo Gd; b) 3D sagittal T2WI FLAIR; c) 3D T2WI; d) 2D axial DWI; e) 3D FLASH (non-IR prep) postGd

Optional: a) Axial proton attenuation; b) Pre- or post Gd axial T1 spin-echo; c) SWI

[4] Optional in Rovira et al and mandatory in Traboulsee et al

[5] Optional in Rovira et al

[6]McGuigan[95] et al and Traboulsee et al recommend FLAIR

[7]Yousry[83] et al recommend FLAIR or T2-w; there is no data suggesting superiority of one sequence over the other in this specific scenario

[8]Spine Traboulsee et al	Mandatory	a) Sagittal T2; b) Sagittal PD, c) STIR, or PST1-IR; d) Axial T2 through lesions
	Optional	a) Axial T2 through complete cervical cord; Gd & post-Gd sagittal T1
Rovira et al	Mandatory	a) Dual-echo (PD and T2-w); b) SE and/or fast SE; c) STIR (as an alternative to PD-w); d) Gd T1-w SE (if T2 lesions present)
	Optional	a) Phase-sensitive inversion recovery (as an alternative to STIR at the cervical segment)

Axial: a) 2D and/or 3D T2-w fast SE; b)GD T1-w SE

[9]Mandatory in Rovira et al and Optional in Traboulsee et al.

Figure 1

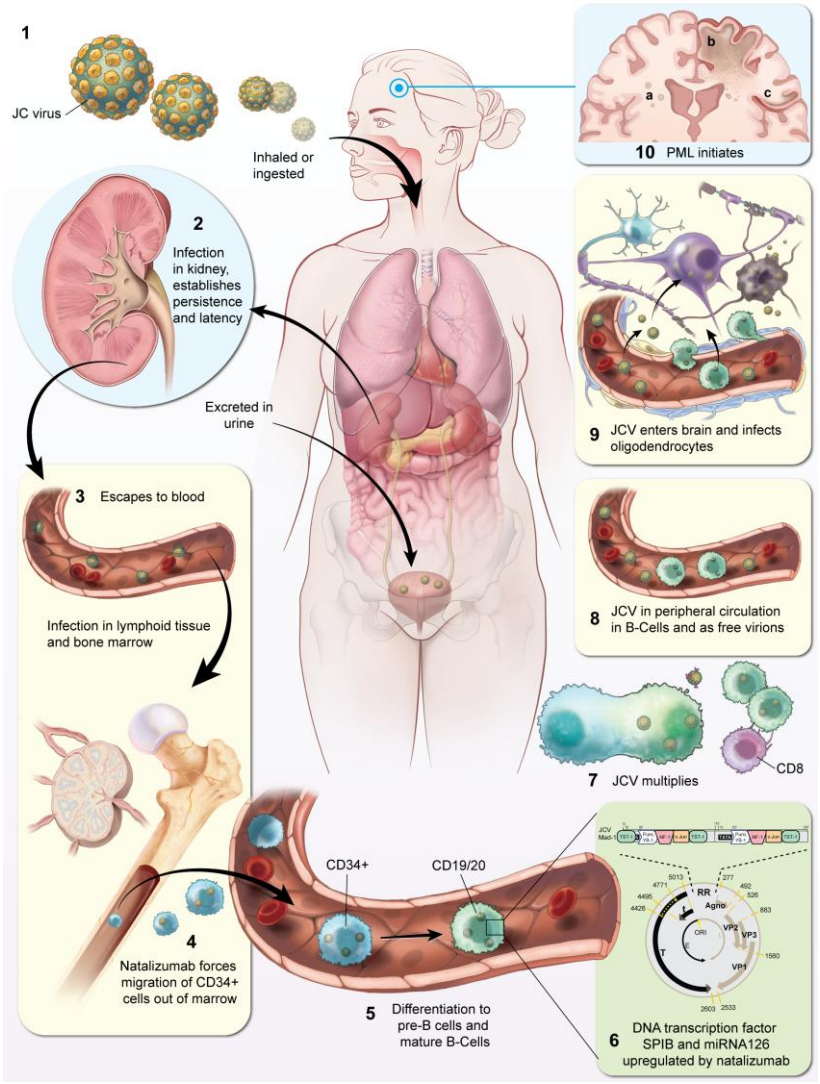
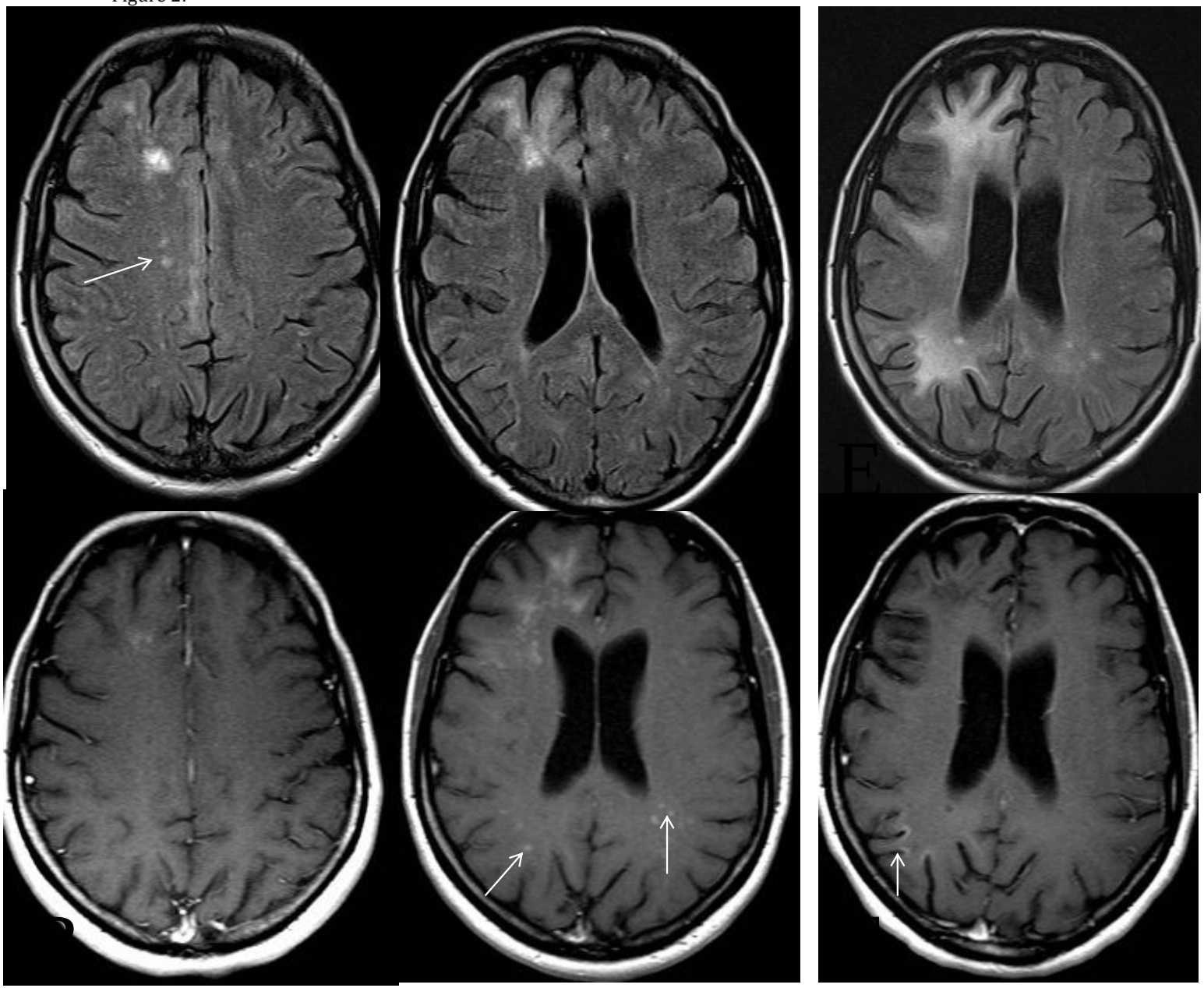

1
JC virus
Inhaled or ingested
2
Infection in kidney, establishes persistence and latency
Excreted in urine
3 Escapes to blood
Infection in lymphoid tissue and bone marrow
4
Natalizumab forces migration of CD34+ cells out of marrow
CD34+
CD19/20
5 Differentiation to pre-B cells and mature B-Cells
6 DNA transcription factor SPIB and miRNA126 upregulated by natalizumab
7 JCV multiplies
CD8
8 JCV in peripheral circulation in B-Cells and as free virions
9 JCV enters brain and infects oligodendrocytes
10 PML initiates
a
b
c

Figure 2:



LEGENDS

Figure 1: Stages of PML pathogenesis

1. Initial infection through ingestion and/or inhalation of virion particles may lead to subacute infection and stimulation of antiviral antibody. No formal study has been conducted however to document these events.

2. In some individuals, approximately 30% globally, JCV infects the uroepithelium of the kidney and establishes a persistent or latent infection as evidenced by excretion into the urine with little if any pathological consequences.

3. JCV may escape into the peripheral circulation in some individuals and spread virions into lymphoid tissues including bone marrow establishing a latent infection that can be reactivated at times of immune suppression or modulation.

4. CD34+ cells in the bone marrow can become infected. Natalizumab forces consistent migration of CD 34+ cells to the peripheral circulation that continues for years during treatment.

5. Some of the migrated CD 34+ cells differentiate in a lymphocyte pathway, predominately in B cell lineage. Some of these cells that may be latently infected use these cells as host for viral multiplication.

6. Both DNA transcription factors like SpiB in the POU2A domain as well as miRNAs are temporally regulated by natalizumab and favor JCV multiplication in latently infected cells. Viral genome may undergo nucleotide rearrangement in the non-coding regulatory region from the urine associated archetype, less pathogenic form to the prototype, PML associated pathogenic form

7. JCV multiplication takes place in these cell phenotypes that may be recognized by CD4 and CD8 immune clearance as well as contributions of anti JCV antibody. Some infected cells escape immune clearance.

8. JCV can remain in circulating B cells, perhaps pre B cells, as well as non-cell associated, free virions in the circulation and traffic to the brain.

9. JCV can enter the brain in infected cells or free virus via hematogenous routes and initiate infection in the target oligodendrocyte. Mechanisms of viral entry are not well documented.

10. PML initiates as virus begins lytic, necrotic oligodendrocyte infection with gradual spreading of virus as evidenced by growing lesions in a multifocal pattern. a)represents MS lesions in PML patients treated with natalizumab; b)cortical white matter lesions with punctate lesions just below that are typical in PML and c)PML lesions in U fibers near the cortex.

Figure 2:

Natalizumab associated PML in an MS patient A, C, E: FLAIR images; B, D, F: Enhanced T1w images

Asymptomatic PML (A and B): Enhancing right frontal lesion with multiple smaller non enhancing punctate lesions (white arrow)

PML and IRIS (C and D): The lesion has enlarged on FLAIR and the enhancing area has increased; note the enhancing punctate lesions bilaterally (white arrows)

Post PML (E and F): Further enlargement of the lesion on FLAIR and presence of T1 hyperintense cortex (white arrow)

INSERT

Key Recommendations:

- Risk biomarkers for PML must be expanded and made more accurate
- Enhanced global data collection on cases of PML should be pursued to inform risk assessment and outcome analysis
- Recommendations for surveillance should be geared to risk profile
- Lower risk patients(<0.9 cases of PML/1000 exposed) should receive routine assessment for MS disease activity as part of disease modifying therapy selection and refinement as well as PML surveillance
- Higher risk patients (>0.9 cases of PML/1000 exposed) should undergo enhanced PML monitoring with more frequent MRI and antibody index assessments
- Updated risk assessments should be available as output from the surveillance network allowing best practices refinement of practice
- Patients with escalating risk factors should change therapy prior to PML detection

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***Reply to Reviewers Comments**

RESPONES TO CRITIQUE OF MANUSCRIPT

We appreciate the careful and lengthy additional suggestions for this manuscript. We also appreciate that the multiple major revisions now appear to be responsive to the opportunity to add to literature helpful to treating physicans contending with PML risk in the setting of MS. Some of the requests are contradictory, and others impossible to respond to without expanding the already large manuscript. We have worked hard to provide meaningful responses that will further enhance the impact and value of our manuscript and hope that now it will be deemed ready for publication.

Detailed responses are shown below.

Manuscript Number: THELANCETNEUROLOGY-D-16-00958R1
Title: A decade of lessons learned: PML pathogenesis and risks associated with therapies for MS
Type: Review

Dear Dr. Clifford,

Thank you very much for submitting your revised manuscript to The Lancet Neurology. We have now received feedback from the original reviewers, and I'm pleased to say that they are positive about your revision. In addition, one further external adviser agrees that the scope and focus of your paper have improved and are rather different from typical PML reviews. Reviewer #1 has made some important suggestions for further revisions, and reviewers #2 and #4 have suggestions for minor changes at this stage. We would therefore like to invite you to REVISE your paper in view of the reviewers' reports (pasted below).

We would like to receive the revised version of your paper by Friday 27th October 2017, but please let me know immediately if you foresee any difficulties with this deadline.

In addition to the referees' comments, my colleagues and I have some editorial recommendations and requirements, listed below, which we hope will help you to complete your manuscript.

General
* If you have not already done so, please submit a copy of your signed author statement form with the final version of your manuscript (available at http://download.thelancet.com/flatcontentassets/authors/tln-author-signatures.pdf), in addition to a completed ICMJE form for each author (available at http://download.thelancet.com/flatcontentassets/authors/icmje-coi-form.pdf).
* Please ensure that the information you give under "Contributors" and "Declaration of interests" at the end of your article matches the information included on the author statement and

ICMJE forms.

* Please provide a panel describing the "Search strategy and selection criteria" you used to identify references for your paper. This should include the search terms used, the dates of the search (eg, papers published from January 2000 to September 2017), the languages of the papers considered, and the way in which you selected the final set of papers. Please include in your manuscript any relevant papers that have been published since your previous revision. In a separate e-mail, I will send you a copy of our recent Article on the risk of natalizumab-associated PML in patients with MS. (THIS IS NOT MEANT TO BE A LITERATURE REVIEW, AND FORMAL SEARCH STRATEGIES WERE NOT EMPLOYED. THE AUTHORS REVIEW AND ASSESS WORLD LITERATURE ON THE TOPIC CONTINUOUSLY. THE REFERENCES HAVE BEEN UPDATED INCLUDING THE LATEST LANCET NEUROLOGY MANUSCRIPT SUPPLIED)
* Please ensure that one qualification (eg, MD or PhD) is included for each author.

Main text

* Please add a 150-word unstructured summary to the start of your paper, which should serve as a taster for the reader, highlighting some of the key messages from the Review without stating explicitly what you will cover (this can be done in your introduction). (the final sentence of the Introduction is the Summary. If moved to the start of the Introduction, we would need to add a few more words, but we would be glad to do that if necessary)
* Although the reviewers have asked you to expand the content of your Review, we need to keep the word count of the main text as close to 5500 as possible, so please aim to reduce the length of the text and avoid repetition, within the text and across the text and display items, where possible. (WE HAVE DELETED SEVERAL OF THE LEAST IMPACTFUL PARAGRAPHS IN ORDER TO CUT THE SIZE OF THIS MANUSCRIPT – EG "CONSIDERATIONS FOR THE CLINICAL DECISION PROCESS" SECTION DELETED, ALONG WITH SEVERAL SENTENCES IN "THOUGHTS ON RISK MEDIATION"
* Please make sure that it's clear from the start of the paper what the main aims of the paper are (eg, highlighting areas of progress and what recent advances might mean, in the short and longer term, for patients with MS). Throughout the paper, please make sure that the link between the different sections is clear (ie, that the flow of the paper makes sense and transitions from one topic to another are not too abrupt).
* Reviewer #1 comments that "it is clear that none of the authors is an expert in MS". Clearly, in revising the paper you have focused on your own areas of expertise, but please be cautious when you consider what new advances might mean in terms of the treatment and management of patients with MS. Note that reviewer #4 feels strongly that alternative (no PML risk) treatments should be found for high-risk patients now that a range of therapeutic options is available for RRMS. (while we indeed have worked in MS related work for decades, we specifically cut from the earlier draft material that sought to frame MS treatment decisions independent of PML… WE HAVE CONSISTENTLY FOCUSED ON PML, ALTHOUGH OTHER REVIEWERS ASKED FOR STRONGER RECOMMENDATIONS REGARDING MS THERAPY CHANGE)

Figures

* The footnotes in the tables are rather unwieldy at present. Please aim to streamline these (eg, could the footnotes for table 2 be included in the table by adding a "Notes" column?).
* Please ensure that all display items (figures, tables, panels) are cited at appropriate points in

the main text; please add a citation for your new panel (key recommendations).
* Please confirm that the figures and tables were produced specifically for the purpose of this paper and have not been published previously. For any figures or tables that have been published previously, please obtain copyright permission from the original publishers and submit any permission letters with your revised manuscript.
* Please submit individual files of each of the figures you wish to include. For photographs (eg, MRI scans), please provide JPEG, TIFF, or EPS, Powerpoint or PDF files. For line diagrams, please provide the figure in the program in which it was originally created. Please do not cut and paste figures from one program to another (eg, Word or Powerpoint).
* Please ensure that the resolution of the figures is at least 300 dpi. Each figure should be provided at final print size, or larger. The width of two columns in the journal is 164 mm; one column is 82 mm.

References
* When revising your manuscript, please note that references in display items should be cited at the point at which the table/panel/figure is mentioned in the main text and in sequence with the other references in the text (ie, for the purpose of reference numbering, display items are considered as part of the text).

When you submit the revised paper, please provide one 'clean' copy and one copy in which your changes are highlighted. In addition, please provide a separate document listing the editorial and referee comments and your replies, point by point. These files must be submitted as MS Word files.

To submit your revised manuscript, please visit The Lancet Neurology's Online Submission and Peer Review Website (EES) at:
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If you can't remember your password, please click the "Send Username/Password" link on the Login page.

After you have entered your account details, remember to click the "Author Login" button. You will see a menu item called "Submission Needing Revision". You will find your submission record there.

Please do get in touch if you would like to discuss any of the points raised in this letter or in the reports. I look forward to receiving the final version of your manuscript soon.

With best wishes,

Dr. Rebecca Craven
The Lancet Neurology

Reviewers' comments:

Reviewer #1: This is a revised version of the original manuscript, which was entitled: JC Virus, PML and Therapies for MS: Weighing the benefits and the Risks. The manuscript has been improved in a number of points, but still the three major aspects: a) historic summary of the still sketchy understanding of PML pathogenesis (see ref 42 for details) with respect to virology and immunology (see ref 18 and 33 and 37 for extensive molecular data on virology), b) use of natalizumab in MS and dealing with the PML risk and what can be learnt from that, and c) how to use MRI as a means of early diagnosis and management, remain in many respects superficial and/or speculative. The title now includes "...risks associated with therapies for MS", but the focus is almost entirely on natalizumab. Many of my original comments still apply, and I will not repeat these here, but list only some new points below:

- In the introduction it is mentioned that the PML incidence in patients, who are on long-term treatment - it is not defined what long term means (24 months or longer is the currently used definition) - and are JCV antibody (ab) positive is at least 1 in 70 (Biogen data for highest risk category of patients and ref 71). It is not clear how the authors come up with this number. There are approximately 150.000 patients, who are being treated or have been treated with natalizumab, and somewhere between 700-800 PML cases have occurred. The majority of patients has received more than 1 year of treatment, and according to the above figure, one would expect 2000 cases or more. The high risk patients are ones with >24 months of therapy, antibody positive and a history of prior immune suppressive treatment. That number is not 150,000. To avoid misunderstandings. I believe that >700 PML cases, >20% of these with fatal outcome and many with severe remaining disability after recovery are very serious, but the numbers that are given are not correct and would not be acceptable in my view. As of Sept 2017 there are 749 cases in Biogen report, so we have indicated >750 cases confirmed.

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- There are still a number of statements like the one in the introduction: "1. the molecular aspects of pathogenesis and cell-specific JCV infection leading to PML that may be generally applicable but also that specifically relate to unique pathophysiological of natalizumab", that are imprecise and difficult to grasp.(see text for changes)

- Another: "It may be time now to consider PML not just as a rare disease but as a ~~substantial~~ significant neurological complication in certain high risk populations". In HIV infection/AIDS, the drop of PML has been dramatic, and while there are a number of treatments (biologicals, small molecules, transplantation), which are accompanied by PML risk, it still remains a very rare to rare condition. Natalizumab-treated MS patients may be the only exception, possibly also CD20-directed B cell-depleting therapies in hematologic malignancies and rheumatoid arthritis. In the context of the latter it is interesting that PML has been associated with CD20 depletion in those conditions, while anti-CD20 antibodies that are widely used in MS have not yet shown any significant risk to develop PML. It would be important to comment on findings like this in a manuscript that tries to link PML pathogenesis to a certain treatment and in a specific disease, i.e. MS. (It should be noted that PML is not a reportable disease so the incidence of PML estimated and not exact. The FDA continues to require may new developed therapies in

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clinical studies that are immune modulatory or lead to immune deficiency have PML risk mitigation plans in place. PML continues to be diagnosed in HIV-1 infected patients but not always reported as is the case in patients with other underlying diseases that place them at risk of PML.

- The statement on page 4: "An increase in antibody titer or index indicates an active infection". As I already stated in my last review, there is no evidence for that.(The operative word here is 'increase' in antibody titer, that denotes a prior lower titer/index that rises over a time period. This is based on many viral infections in which there was an 'acute' serum sample taken during initial clinical symptoms and the later when symptoms gradually subside or 'in convalesence'. This is fundamental virology, recently exemplified in Zika infection and before in West Nile infections). In fact it is not known what a high index means. (published data describes patients with a high index >1.5 have a greater risk of PML. See current Lancet Neurology paper from Biogen statisctians with more details). Patients, who show shedding of JCV into the urine - approximately 40-50% (perhaps more like 30%)of all JCV-infected individuals - also must have active infection, but it is not known if this is linked to a high JC ab index, whether non-shedders can become shedders, and what the pathological - if there are any - consequences of viral excretion are. The reasons why some patients start with a high JCV ab index and usually stay high, while others show a low index after seroconverting, i.e. from the beginning, and then usually stay low are also not known. If high and rising antibody indices indicated active infection or higher viral loads or change of viral types from wild type to PML strains, then one would expect that some data for one of these, e.g. higher viral DNA load in the blood, would have been described, but I am not aware that this is the case. It is of course also possible that it has not been examined yet, but at any rate, the meaning of high JCV ab indices and how they may predispose to PML are not known. The authors therefore should either be more specific with their statements or avoid them. The text includes the current knowledge of this area. Further discussion on this topic may be worthy of a more specific review.

- Further down: "the VP1 gene can be hypervariable...". Hypervariable means that a gene is frequently mutated, as is for example the case in immunoglobulin genes after rearrangement and affinity maturation. My understanding is that mutations in VP1 are overall - when compared to capsid proteins of other viruses - rare and that only relatively few mutations have been described in VP1 in PML- and granule cell neuronopathy (GCN) variants of JCV. (The VP-1 gene is hypervariable which is the basis for the 'typing system' of JCV variants that was described in the mid 1990s, see reference27 and references therein. Also the Type of JCV variants, based on VP-1 sequences have a clear geographic distribution with a number of epidemiological studies identifying regional JCV infections.

- "So, it is not surprising that any one PML patient may have multiple representations of VP1 at any one time". Again, what is the evidence for this statement? While in theory this is a possibility, there is very little data on it. A patient, who develops PML should have wild type (wt) and PML variant at the same time, but presence of multiple PML variants is certainly not a frequent and documented phenomenon. (see ref 30 on deep

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sequencing of JCV DNA in PML patients as well as the JCV Compass paper Jensen, P et al J Leukocyte Biol 65: 1999 and Jensen P et al J NeuroVirol 7: 2001). At least one of the two papers that are cited in the next sentence does not provide evidence for that, but only mention a GCN variant, but not presence of multiple PML/GCN variants. (There are variations in the VP-1 gene associated with JCV/GCN at a region near the 3' intergenic region of the genome. There is no unique variation in this region that describes GCN but variations over a number of different nucleotides (ref 15). With a limit of 100 references, it is difficult to list all the details and references on these topics. For a more complete review, see Ferenczy M et al in Clin Micro Review,25; 471-506, 2012.

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- The description as to which immune components are important in maintaining control of JCV infection or are involved in recovery from PML is very sketchy. If one considers the spectrum of conditions that lead to PML - ranging from different types of hereditary immunodeficiencies over drugs with more or less specific effects on the immune system to autoimmune diseases with different pathogenetic mechanisms - it is clear that neither CD4+ or CD8+ T cells nor antibodies and B cells alone are important, but that the issue is more complex. The authors brush over this area in a very superficial way that does not take into account the substantial progress that has been made in recent years. See ref 29 for a more detailed discussion on immunity. This paper is from Roland Martin's lab in Zurich who is a highly regarded neuroimmunologist.

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- What is meant with: "... temporal upregulation of factors that highly favor JCV growth"? The szenario that transcription factors and recombinases of B cells may participate in mutation is reasonably supported, but that natalizumab or factors associated with it favor JCV growth has not been described to my knowledge. See reference 41 from L. Kappos group in Basel and Lindberg R from that group as well as ref 40, 42.

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- After describing the JCV biology and possible factors that may lead to PML under natalizumab or in general the authors jump abruptly to MRI. Not even the diagnostic requirements for PML are introduced before that. (A transitional sentence was inserted to smooth this transition. Because of space restrictions, we had to cut out an entire section on diagnosis from earlier drafts, but this is well covered in other reviews)

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- The statement, "Non-specific white matter lesions are common, and in MS patients lesions may be part of the underlying disease", is again imprecise or wrong. Lesions in certain typical locations (which overlap with those, where PML occurs) are characteristic for MS and the most important diagnostic finding. They also pose a major problem in identifying early PML lesions in a brain that shows often a high lesion load already. Having worked and published over the last 20 years on the various aspects of the imaging of MS (diagnosis, visualisation, outcome measures) we are of course aware that there are typical patterns of MS lesions. However, the specificity is limited as they can occur in healthy individuals, reflect silent progressing disease (small vessel disease or other inflammatory or other disorders. In Yousry et al. 2012, we highlighted the problem of identifying PML lesions in a brain with MS lesions. To improve clarity, we have modified as follows: "Small lesions can be difficult to differentiate from MS lesions especially when there is a high lesion load (ref 61; Yousry et al. Annals 2012)"

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- In many parts of the manuscript, it is clear that none of the authors is an expert in MS. To include this would certainly have helped. (TY member of MAGNIMS)

- "It is fundamentally untenable that the specifics of type and duration of prior immunotherapy is of little consequence in determining risk on a biologic basis, yet this is at present a monolithic consideration". This sentence is very cryptic. Such statements do not help, but only confuse the reader. (We think this is quite a precise statement. We could say that based on widely varying mechanisms and potency of immunotherapy, they are very likely to have quite different impact on the immune system or evolution of JC virus, yet they are at present considered as having only one possible contribution… this takes more words and seems less impactful than our current way of saying this…)

- "If a therapy has the possibility to halt disease progression in a majority of cases, but there is a slight risk of death (approx. 25% of the small number of PML cases) or instead, the risks of therapy sound reasonable to an individual with a risk affirmative to living." First, the sentence is incomplete. (modified to help the reader see full sentence) Second, the authors swing between dramatizing (more than 1 in 70 patients treated with natalizumab have suffered from PML; in the introduction) to trivializing the risks of natalizumab in this sentence here. (we are satisfied the reviewer now understands the tension that presents itself to the patient and physician. This reviewer is not in the camp of "risk affirmative" but surely must recognize that superior MS management might balance risk of PML for some with worsening disability (and death?) from MS) No other MS treatment has caused remotely as many deaths as natalizumab, and that in a disease that affects young individuals without a major compromise of life expectancy. > 700 PML cases is a substantial number, and, as already indicated before, if patients do not die from PML they often have severe remaining disability. Furthermore, a series of other drugs is now available that are as effective or more effective than natalizumab. (these drugs were not available at the time we wrote this, and their track record is very short and incomplete at present) Finally, if one considers a new measure of MS disease activity, NEDA = no evidence of disease activity based on relapses, disability accrual and MRI findings, then the fraction of MS patients, who fulfill NEDA criteria after two years, which is a short period in the course of MS, is only approximately 45%. Again, it would have helped if an expert in the field of MS had contributed to the manuscript. As an aside, NEDA is referred to as NEAD further down. (please note this manuscript was altered to focus on PML and not on MS treatment at the request of the editors)

- "Alternatives to lymphocyte counts might include serial antibody measurements, or measurements of circulating JC virus." Neither of these would be meaningful from my perspective, or at least there is no data to suggest that. Furthermore, the authors should have at least mentioned a recently described marker, CD62L expression. (This is now mentioned and referenced)

- The information in table 3 for PML surveillance is in part helpful, but too complicated. Since the spine MRI is not routinely done for PML surveillance, it could be left out. The brain MRI should consider both, continued monitoring for new MS activity and at the same time indications for early signs of PML. The information, which MRI sequences and how often one should scan,

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could be conveyed in much simpler and easier to grasp form. In the higher risk strata, the information how often JCV ab indices should be measured are not stated.

We have stated in the legend: "[1]Spine MRI is not indicated for PML monitoring, but may be used in monitoring MS disease activity, or if neurological exam suggests possible spinal cord localization of pathology". We suggest this table to be used for surveillance of PML; this surveillance is integrated in the routine and regular scanning for MS activity, which does include spine imaging. Leaving out the spine will make it incomplete, thus limiting its practical usage. We prefer to keep the table because we suggest it to be used in the routine setting and to not affect the word count.

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- Despite my mentioning it already in the first version, Fig. 2 on page 30 still does not have labels on the various panels, i.e. it is not clear what A, B, C, etc. is.

Comment [TY1]: I don't know what he means or wants

The lergend states: "Natalizumab associated PML in an MS patient A, C, E: FLAIR images; B, D, F: Enhanced T1w images". The image when viewed on the computer screen has the letters, but on printing this did not show for some reason, so this reviewer must have printed the copy used for review. ~~We will try to assure that a printed version also shows the letters.~~The letters and arrows now print on our printer without difficulty as formatted.

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- The manuscript does not mention treatments of IRIS including the CCR5 inhibitor maraviroc, which would be of interest, and neither are the previously proposed treatments, mirtazepine, mefloquine and others discussed. Management has been reviewed elsewhere and is referenced. However, we agree with this reviewer that having completely cut the management discussions is a shortcoming in the manuscript. To address this we have added 5 sentences with appropriate references to frame the recommendations and controversies of PML and IRIS management including several 2017 references to guide the interested reader.

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Reviewer #2: Dr. Clifford and colleagues review the current state of knowledge about the pathogenesis of PML with an emphasis on what we have learned from the experience with natalizumab. They also expand on existing recommendations regarding risk mitigation with the use of natalizumab.

Comments:

1) The discussion of immune protection against JCV and PML development makes little comment on the potential role of neutralizing antibody. For completeness, a sentence or two regarding its potential role should be included. Bottom of Page 5 now has sentences on role of antibody in neutralizing JCV infection.

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2) The authors cite Buckle's paper in which the investigators were unable to amplify JCV from MS brains studied. On the other hand, there have been a fair number of papers that have demonstrated JCV DNA in low copy numbers in the brains of individuals without PML. This should be noted, though the authors may want to comment on the absence of evidence of viral replication in these brains. Bottom of page 6 and top of page 7 now has sentences addressing

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JCV latency in brain and includes a NEW reference (Tan CS, Ellis LC, Wuthrich C et al. JC virus latency in the brain and extraneural organs of patients with and without progressive multifocal leukoencephalopathy. J Virol. 2019; 84:9200-9209.

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3) The authors posit that evolving MRI lesions with PML may occur in tandem with increasing antibody titers. While the Gorelik paper (Ann Neurol 2010) was unable to demonstrate a correlation between the JCV antibody index and levels of JCV urinary shedding, a recent publication (Berger JAMA Neurol 2017) showed that higher antibody levels were associated with higher copy numbers of virus in the kidney and broader distribution of the virus systematically. The latter is supportive of their contention regarding rising antibody index levels correlating with PML evolution. Reference to this recent manuscript was added.

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4) The authors might want to include data from studies of MS patients' and treating physicians' risk tolerance in their discussion of the clinical decision process. This is an important topic that the manuscript can't support full discussion around, but we have added a sentence and current reference (Kramer et al 2017) to give the interested reader an entrance to that literature.

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5) In Table 2, the authors should clearly define what they mean by "duration". Excellent point, that we have addressed by more specifically defining the term "DURATION".

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6) The "punctate lesions" are included only in the PML IRIS category, but in this reviewer's experience, it has been observed in PML without IRIS. Is there data to suggest it is specific for PML-IRIS? If so, include it.

We suggest that the occurrence of the punctate lesions in natalizumab treated patients is an indication of IRIS. We haven't found reports describing them in non-MS patients, but although unusual wouldn't exclude that this could happen, particularly in more recent HIV cases occurring during immune reconstitution. Our consideration of this includes recognizing that IRIS as defined by enhancing MRI lesions is a crude measure of actual inflammatory responses in the brain (particularly where steroids have been used in mistaken efforts to treat MS exacerbation or in pre-emptive IRIS therapy post PLEX). We suspect that the presence of these lesions may be a more sensitive indicator of IRIS than enhancement, and it is because of this that we think they may be particularly important.

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7) In Table 3, the suggestion is that the JCV antibody be obtained every 6 months. In this reviewer's institution, it is done every 3 months in all patients and once positive, is regarded as positive, regardless of the nature of subsequent tests. They might consider offering a range of 3-6 months. We recognize that recommendations could be graded. Given the very small rate of change, and our understanding of the duration of latency of JC virus leading to clinical PML, we believe q 6 mo is adequate, but would have no objection (save cost) to more frequent testing. We prefer to recommend what to us is a more practical and rational frequency for now.

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Reviewer #3: This major revision improved the manuscript significantly. Esp. the new tables and figures are extremely helpful and provide relevant information to the reader.

Thanks!

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Reviewer #4: The authors have prepared a restructured manuscript, focussing on their key expertise and mainly defining natalizumab (NAT)-associated PML. It is a true pleasure to read the well developed virological part.

on page 9 (in landscape Format) 'asymptomatic PML' is used as subheading - I would consider to denote 'presymptomatic JCV infection' indicating that PML will most probably unfold if Treatment with NAT is not stopped.
same page lane 5 Ref 101 McGuigan has to be incorporated

Agree to change both in heading and Table 2 – a fine point but reasonable.

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on page 7 pp early MRI is handled. Dr Yousry was instrumental in examining the MRIs from the first pivotal NAT study patients. I wonder why he does not comment whether Long - Standing NAT Administration often afflicts brainstem and infratentorial structures with PML

on page 15 conclusion, paragraph 3: I would clearly support an alternative view, namely switching high-risk NAT-treated MS patients to alternative meds. The easiest Approach is certainly Bcell depleting ocrelizumab which is approved in US and awaited in Europe. But also fingolimod, alemtuzumab and daclizumab may qualify as serious follow-up medication post NAT. When you have seen dozens of NAT PML MS patients and their partially poor outcomes, there is a clear need to stop this Russian roulette. We were criticized in making MS therapeutic recommendations, so had removed this. I agree that the viable options call for changing therapy when risk is clearly rising in patients. A sentence was added just before the description of alternate MS therapies recommending routine switching to alternate therapies as risk is increasing relative to natalizuamb.

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In central Europe a vaccination using recombinant JCV is under regulatory approval - it may be worthwile to comment on this in the conclusion. Alternatively key peptides with the immunogenic epitopes have been developed.

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The development of either antibody directed or T cell mediated immunization/vaccination is now considered more relevant. However, prophylactic or therapeutic use of vaccination against directed JCV peptides for wxample bring challenges to define the target groups either as those at PML risk based on underlying disease and their therapies or the population in general.

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Figure 1

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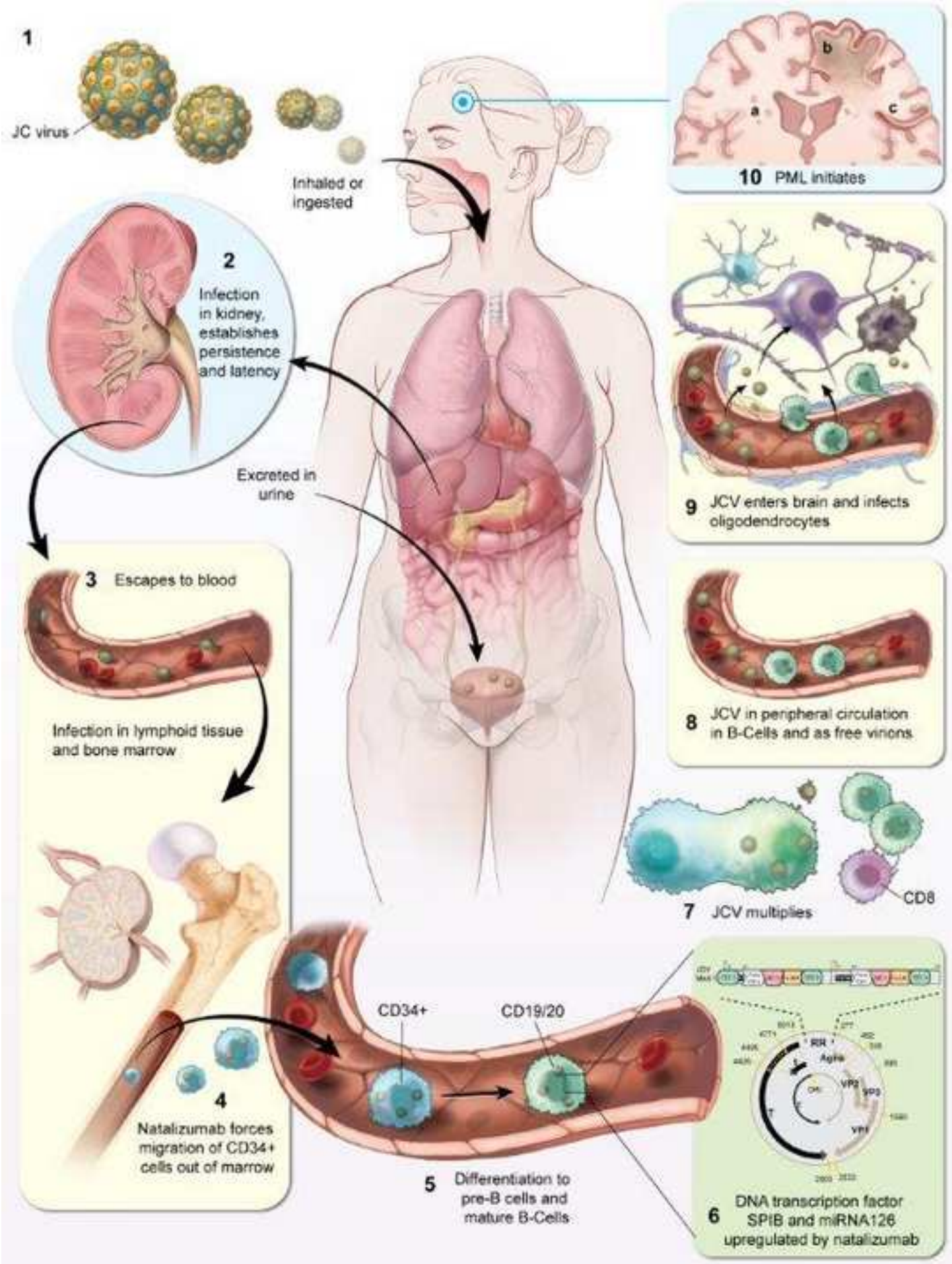
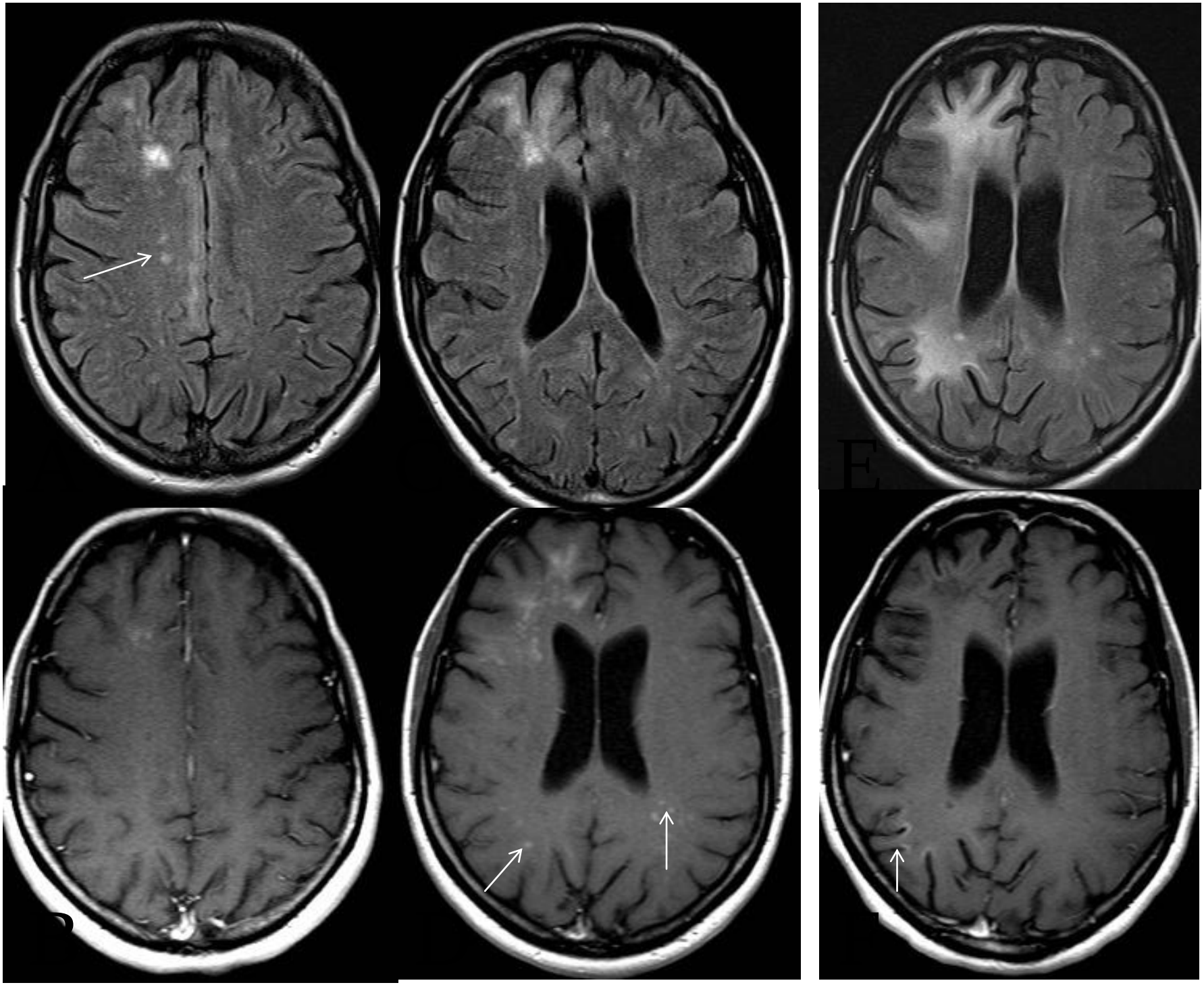


Figure 2 - Letters show in Word, not PDF
Click here to download Figure: Figure 2 with legend_2017v.docx

Figure 2:
Natalizumab associated PML in an MS patient A, C, E: FLAIR images; B, D, F: Enhanced T1w images
Asymptomatic PML (A and B): Enhancing right frontal lesion with multiple smaller non enhancing punctate lesions (white arrow)
PML and IRIS (C and D): The lesion has enlarged on FLAIR and the enhancing area has increased; note the enhancing punctate lesions bilaterally (white arrows)
Post PML (E and F): Further enlargement of the lesion on FLAIR and presence of T1 hyperintense cortex (white arrow)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