Abstract

JC polyomavirus (JCV) establishes an asymptomatic latent and/or persistent infection in most of the adult population. However, in immunocompromised individuals, JCV can cause a symptomatic infection of the brain, foremost progressive multifocal leukoencephalopathy (PML). In the last two decades, there has been increasing concern among patients and the medical community as PML was observed as an adverse event in individuals treated with modern (selective) immune suppressive treatments for various immune-mediated diseases, especially multiple sclerosis (MS). It became evident that this devastating complication also needs to be considered beyond the patient populations historically at risk, including those with hematological malignancies or HIV-infected individuals.

We review the clinical presentation of PML, its variants, pathogenesis, and current diagnostic approaches. We further discuss the need to validate JCV-directed interventions and highlight current management strategies based on early diagnosis and restoring JCV-specific cellular immunity, which is crucial for viral clearance and survival. Lastly, we discuss the importance of biomarkers for diagnosis and response to therapy, instrumental in defining sensitive study endpoints for successful clinical trials of curative or preventive therapeutics.

Advances in understanding PML pathophysiology, host and viral genetics, and diagnostics, in conjunction with novel immunotherapeutic approaches indicate that the time is right to design and carry out definitive trials to develop preventive options and curative therapy for JCV-associated diseases.

Keywords: PML, JCV, diagnostics, risk stratification, clinical studies, biomarker

Background

Progressive multifocal leukoencephalopathy (PML) is a potentially life-threatening disease of the brain in the immunocompromised host caused by lytic infection of white and grey matter cells by JC polyomavirus (JCV), also referred to as JC virus or human polyomavirus type 2. The mortality rate can be high, ranging from 20-90%, depending on the underlying condition [1]. Although no class I evidence for JCV-specific therapy is yet available [2], the recent encouraging application of a variety of immunotherapeutic approaches, including virus-directed T cell therapies and check-point inhibitors, creates the justified hope that the development of such therapies may advance to formal testing in clinical trials [3].

This review provides a brief overview of the current understanding of PML and its clinical variants. It focuses on the pathogenesis, diagnostic tools, and potential biomarkers of response to therapy. Based on current literature, we conclude that candidate surrogate biomarkers need to be optimized and validated to facilitate diagnosis of PML, ideally at a pre- or asymptomatic stage. These and additional biomarkers should further be evaluated as potential endpoints of response to therapy in studies aiming at approval of JCV-directed curative and preventive therapies. The biomarkers discussed include standardized brain magnetic resonance imaging (MRI) protocols, measures of JCV viral variant load in cerebrospinal fluid (CSF), and biomarkers of humoral and cellular JCV-directed immune responses.

Main text

JCV-associated diseases

PML and common predisposing conditions

A prerequisite for the development of PML is a weakening of JCV-directed cellular immunity [4]. Accordingly, PML was first described in patients with hematological malignancies, such as Hodgkin's disease and chronic lymphocytic leukemia [5], which today still represent at least 10% of PML cases [6].

From a historical perspective, two more consecutive epochs of PML can be identified based on the frequency of PML-related publications (figure 1). First, the emergence of the human immunodeficiency virus (HIV)- and acquired immunodeficiency syndrome (AIDS) pandemic in the early 1980s increased the number of publications. PML is listed among the AIDS-defining conditions, and infection with HIV to date remains the most prominent underlying condition for PML, accounting for 50-80% of all PML cases [6].

From 2005 on, the interest in PML-related research was triggered by the occurrence of PML in people with multiple sclerosis (MS) treated with the monoclonal antibody against α_4-integrin, natalizumab (Biogen, Cambridge, MA, USA). Compared to other approved modern disease-modifying therapies (DMT), natalizumab is associated with the highest risk for developing PML, with an overall incidence of 3.1 in 1000 treated individuals. The risk of

developing PML notably increases beyond the second year of therapy, in individuals previously treated with classical immunosuppressants (such as azathioprine, mitoxantrone, cyclophosphamide), and those with high JCV specific serum antibody levels as determined by the Stratify-JCV™ serological testing and reported as "index values". Accordingly, the risk is lowest among seronegative individuals (around 1 per 10.000), and highest (around 1 per 100) among individuals treated for more than 5 years and with Stratify-JCV™ index values above 1.5 ([e81]). Extended interval dosing (more than 4 weeks) of natalizumab may reduce but does not eliminate the risk of PML[7,8], and seroconversion rates (negative to positive) explain the recommendation for bi-annual retesting minimum in the JCV seronegative.

The LFA1-targeting monoclonal antibody efalizumab, previously used for the treatment of psoriasis, was withdrawn from markets in 2009 due to the occurrence of PML cases, indicating that integrin-targeting DMT specifically increase PML risk [9]. Following this observation, PML was added as a possible complication of several DMT in immune-mediated diseases, although with far lower overall risk estimates (<1 in 10.000). DMT used in the field of neurology with labeled warnings of risk of PML include dimethylfumarate, fingolimod and other S1P-modulators, ocrelizumab, rituximab (off-label), ofatumumab and inebilizumab, even though, as of December 2022, no confirmed cases of PML have been reported for ofatumumab (in MS) and inebilizumab (for neuromyelitis optica) ([e82-e87]). Altogether, people with MS treated with DMT, foremost natalizumab, are currently estimated to account for around 5% of all PML cases[6]. As immune competence can be restored by drug discontinuation, DMT-associated PML may have a better prognosis in terms of survival and disability as compared to PML in individuals with hematological malignancies or in HIV patients prior to the availability of combined antiretroviral therapy (cART, for additional information comparing HIV- and natalizumab-associated PML see table 1).

Rare genetic disorders resulting in inborn errors of immunity, commonly grouped as primary immune deficiencies, can also lead to an increased risk of PML [10]. Moreover, PML is seen in post-transplant patients, individuals with sarcoidosis and rheumatological diseases, or cancer patients treated with targeted therapy (including rituximab, brentuximab, ofatumumab, alemtuzumab, obinutuzumab, ibrutinib, belimumab, idelalisib, and CAR T cell therapy), and has also been rarely noted in higher-aged individuals without apparent immunosuppression [11–15].

Granule cell neuronopathy and rare variants of JCV-associated disease

Granule cell neuronopathy (JCV-GCN) defines a distinct disease entity of JCV-associated disease with an infection primarily of granule cells of the cerebellum. Clinical and neuroradiological signs include ataxia and progressive cerebellar atrophy [16]. Specific viral-genetic changes may explain the tropism for cerebellar granule cells [17]. JCV-GCN can occur

distinct from PML, but also simultaneously with PML, as observed in around 5% of PML cases [18]. Other variants of JCV-associated disease may exist but are not well defined, including JCV-associated encephalopathy (with predominant involvement of the cortical grey matter and infection of pyramidal neuronal cells), meningitis, or nephropathy [6].

Pathogenesis of PML

Transmission and reservoir

JCV establishes a persistent asymptomatic infection in 50-80% of the adult population, showing increasing seroprevalence with age. Illustrating this lifetime exposure risk, in people with MS anti-JCV antibody seroprevalence was reported at 47% among 15–29-year-olds, 59% among 40-49-year-olds, and 64% for individuals older than 60 years of age [19]. JCV transmission and the route of primary infection are not well understood, however this most likely occurs via a fecal-oral route during childhood [1,4,6]. It has been assumed that following a period of asymptomatic viremia, JCV establishes latent and/or persistent infection in the kidney, lymphoid organs, bone marrow, and possibly the brain and elsewhere in the body. In association with immunosuppression, JCV can reactivate from sites of latency or persistency, undergoing viral genetic changes to become a pathogenic neurotropic virus. Thus far, it is unclear where the disease-associated JCV reservoir is located and how JCV acquires the relevant genetic changes. Several potential mechanisms of viral trafficking into the central nervous system (CNS) have been proposed, with conclusive evidence still lacking [6].

JCV DNA excretion in urine is commonly observed in around 30% of healthy individuals. However, PML-type JCV genetic variants (see below) have not been found in urine, and excretion of JCV in urine does not appear to increase the likelihood of developing PML in HIV-infected individuals, arguing against uroepithelial cells as a relevant reservoir for JCV from which PML-development is initiated [20,21]. In the context of natalizumab-associated PML, the bone marrow has been proposed as a potentially relevant reservoir, as hematopoietic progenitor and B cell numbers increase in peripheral blood during natalizumab therapy, and the B cell DNA recombination machinery might allow the virus to acquire pathogenic viral genetic changes [22,23]. However, no further evidence has been generated supporting this hypothesis [24].

Besides entering the brain as a free virus or within B cells, JCV might use extracellular vesicles (EVs) to relocate to the brain. Viral particles can be released by infected cells within or attached to EVs, thereby influencing viral tropism by allowing viral receptor-independent cell entry and permitting immune escape of virus hiding within protective EV membranes. A clear role of EVs in JCV spread and PML pathogenesis has not yet been established. However, JCV virions have been found in vitro associated with EVs when infecting human fetal glia-derived

SVG-A cells and immortalized human choroid plexus epithelial cells [25,26]. More recently, JCV DNA has been detected in EVs in the plasma of HIV-infected human subjects [27].

Viral genetics

The JCV genome is a double-stranded, circular DNA molecule of ~5100 bp in size, encoding six proteins, which can be divided into three segments: early and late genes and a non-coding control region (NCCR). Proteins encoded by the early genes, comprising the small and the large T-antigen, are involved in viral replication and the transcription of late genes, specifically capsid proteins VP1, VP2, and VP3, as well as the regulatory agnoprotein. NCCR includes the origin of replication and sequences that control the transcription of both early and late genes. Different JCV genotypes can be determined by the length and sequence of the viral genome based on the NCCR or analysis of sequence variations within the major capsid protein gene, VP1, and the T-antigen [28].

Rearrangements of the JCV genome in the NCCR have been identified as a pre-requisite for establishing PML. The wild-type virus detected in the urine of healthy, asymptomatic individuals lacks rearrangements in the NCCR region. Thus, the rearranged NCCR sequences define the PML-type JCV, detected in CSF, blood, and cerebral tissue of PML patients. Rearrangements occur due to partial duplications, deletions, and combinations of both and are believed to increase replicative ability [29].

JCV-PML genetic variants frequently have point mutations in the VP1 region [30]. Point mutations in the VP1 gene cause conformational changes of the VP1 protein on the viral capsid and can alter binding ability and cellular tropism, possibly impeding viral neutralization and facilitating the infection of brain cells [20,21,31].

Host genetics

While predisposing conditions causing profound immune suppression are essential for developing symptomatic JCV-associated disease in most PML cases, only a small proportion of individuals at risk will develop it. It is therefore hypothesized that host genetics influence susceptibility to PML. In support of this, JCV-infection is strongly associated with human leucocyte antigen class II variants [32]. Among non-HLA genes that might regulate susceptibility to PML, blood group genes appear to be relevant candidates [33]. Nineteen rare germline PML risk variants that affect 19 genes related to immune function have been identified using whole-exome sequencing [34]. Furthermore, polymorphisms of the tumor suppressor protein p53, possibly able to modulate viral gene expression, could also increase PML susceptibility [35].

Diagnosing PML

In 2013, a consensus statement for PML diagnostic criteria was published by members of the Neuroinfectious Disease Section of the American Academy of Neurology (AAN). Diagnostic classification distinguishes between definite, probable, and possible PML based on compatible clinical presentation, demonstration of the lesion(s) suggestive of PML on brain magnetic resonance imaging (MRI), and detection of JCV. The direct detection of JCV is the prerequisite for a definite diagnosis of PML and can be achieved either by JCV DNA in cerebrospinal fluid (CSF) or histologically (figure 2) [36].

Clinical findings compatible with PML

PML might commence as a focal leukocortical disease or as an early multifocal disease. Oligodendrocytes and astrocytes are typical target cells of symptomatic JCV infection, but growing evidence suggests early infection of granule cells of the cerebellum during GCN or cortical neurons contributing to encephalopathy [37]. Accordingly, PML can have a variable initial clinical presentation, ranging from subacute cognitive and neuropsychiatric alterations (frequency 36-54%, range reflects different patient cohorts studied), visual disturbances (e.g. hemianopsia, frequency 19-41%), motor (frequency 33-45%) or sensory (frequency 7-19%) dysfunctions, coordination and gait difficulties (frequency 13-35%), to epileptic seizures (frequency 5-14%) [36]. Unlike in MS, the optic nerve and the spinal cord are typically not involved (figure 2). Differences in frequencies of symptoms reported at presentation in relation to underlying diseases (eg. HIV - vs. natalizumab-associated) or patient outcome cannot be reliably compared across-studies, considering variability with regards to study quality and design. Systematic studies that associate symptoms at presentation with prognosis are lacking, but the clinical expert impression is that early infratentorial involvement might associate with worse prognosis.

Magnetic resonance imaging findings in PML

Changes on MRI highly suggestive of PML have been typically described as T2 hyperintense lesions located in the juxtacortical and deep white matter. The lesions do not respect the border between the white matter and the adjacent cortical grey matter, and therefore, may present as leukocortical lesions. Depending on the progression of the disease, the lesion may appear as T1-isointense lesions (early in course) or hypointense lesions (advanced disease), due to irreversible demyelination, compared to the non-affected healthy appearing white matter. In PML lesions with active viral replication, areas with hyperintense signal intensity on diffusion-weighted imaging, particularly at the border of the PML lesion(s), can be observed, reflecting swelling of oligodendrocytes due to lytic infection. These areas can show various apparent diffusion coefficient (ADC) values (low, normal or high values)[38,39].

Multiple small T2 hyperintense lesions (with or without contrast enhancement) in the vicinity of the primary PML lesions can be frequently identified, an imaging pattern labeled as a "punctuate pattern" or "milky way appearance" [40,41] (figure 3).

Depending on the amount of inflammatory activity or antiviral immune activity, initial imaging findings in PML differ from case to case. On a group level, HIV-associated PML in cART naïve individuals or cases of PML due to hematological malignancies with minimal JCV-directed cellular immune function are unlikely to demonstrate contrast enhancement at first presentation, termed classical PML in literature. In contrast, in HIV-associated PML treated with cART or DMT-associated PML, best documented for natalizumab-associated PML in people with MS, the immune system likely remains partially functional, and even the earliest MRI may show evidence of active inflammation with gadolinium enhancement and perilesional edema [40,42].

CSF findings in PML

Along the current diagnostic criteria, detection of JCV DNA by PCR in CSF establishes the diagnosis of definite PML in individuals with consistent clinical and imaging findings not better explained by other disorders [36]. However, particularly in cases of PML related to DMT use, CSF JCV DNA load is often below 100 copies/mL CSF, resulting in false-negative results by many commercially available quantitative molecular assays, which commonly have lower limits of detection above this threshold (often about 100-200 copies/mL CSF) [43,44]. Due to a wide variability of sensitivity and specificity of available JCV DNA PCR assays, interpretation of accuracy of PML diagnosis needs to account for the method used. AAN diagnostic criteria claim that sensitivity of newer ultrasensitive techniques that detect up to 10 copies/ml CSF is >95% [36]. However, other publication in smaller cohorts of HIV-associated PML observed a drop in sensitivity from 89.5% in the pre-cART era to 57.5% in the cART era, likely related to assays with a higher lower limit of detection, but also related to cohorts with low copy numbers of JCV DNA in CSF [45]. Therefore, undetected JCV DNA in CSF does not preclude the diagnosis of PML and repeat and reference laboratory testing or brain biopsy may be needed to definitively establish diagnosis.

In the future, improvements and new developments in PCR techniques might help overcome the problem of limited PCR sensitivity, including more sensitive digital droplet PCR techniques [46]. The detection of elevated anti-JCV specific immunoglobulin G (IgG) antibodies in CSF with evidence of intrathecal production has been observed in cases of PML, suggesting the so-called CSF JCV-specific antibody index that corrects for disturbances of the blood-CSF-barrier function may be a promising future added tool for diagnosis of PML, still requiring independent and ideally prospective validation [47].

Standard clinical CSF parameters are most often within the normal range in individuals with PML. Abnormalities are most commonly related to the underlying condition predisposing to PML, e.g., explaining slightly elevated leukocyte cell count levels and blood-CSF-barrier dysfunction in individuals with AIDS or the detection of oligoclonal bands in individuals with MS (figure 2).

Histopathology findings in PML

Definite diagnosis of PML can be established by neuropathologic demonstration of the typical histopathologic triad, which are demyelination, bizarre astrocytes, and enlarged oligodendrocyte nuclei, together with tissue detection of JCV (figure 4). This latter can either be achieved by immunohistochemistry, in situ hybridization, electron microscopy [36], or by tissue PCR detecting JCV DNA (figure 2). In classical PML, few lymphoid cells and numerous virally infected cells are found. In contrast, in inflammatory PML, as for example observed in natalizumab-treated patients, pronounced inflammation is noted and often only few virally infected cells with enlarged oligodendrocyte nuclei are present [48].

Challenges in diagnosing PML early in disease course

The AAN consensus PML diagnostic criteria are widely in use in clinical routine. Nevertheless, PML diagnosis is still often delayed [12,43], negatively impacting the outcome. Several reasons contribute to this diagnostic delay:

To begin with, PML is a rare disease, such that awareness among physicians – even among neurologists and general radiologists - is limited. Furthermore, PML-specific symptoms do not exist. Nevertheless, in the context of patient history and preexisting risk factors, specific symptoms should raise a strong suspicion of PML. For example, new onset retrochiasmal visual disturbance, such as hemianopsia, in younger individuals with an increased risk of developing PML (i.e. HIV-infected or JCV-seropositive MS patients treated with natalizumab) must lead to appropriate diagnostic workup using MRI and CSF studies. Therefore, discussing PML in educational materials and management guidelines for certain conditions, such as AIDS or MS, is essential to increase awareness.

A more prominent role of MRI in diagnosing PML could potentially allow earlier diagnosis of PML in some patients. The knowledge of an increased risk of developing PML during therapy with natalizumab has led to risk mitigation strategies, including MRI screening for PML using sensitive protocols and imaging every 6 months or even more frequently. These recommendations were based on the observation that PML can be detected in a subgroup of patients with natalizumab-associated PML before developing clinical symptoms, termed asymptomatic or pre-symptomatic PML [43,49]. Furthermore, early detection on MRI at an asymptomatic stage may lead to a better outcome and survival compared to patients already

symptomatic at the time of diagnosis [50]. One study retrospectively applied the current PML diagnostic criteria to a population of natalizumab-associated PML frequently screened for PML using MRI and compared the results to an alternatively proposed case definition, exposing limited sensitivity of both in this setting [51].

As discussed above, current PML diagnostic criteria would possibly be more sensitive in a real-world clinical setting if highly sensitive JCV PCR protocols with optimized pre-analytic procedures were available for all individuals suspected of PML. This highlights the need for improved molecular assays for JCV in biological fluids, and broad access to these already at the time of first testing in local laboratories, to avoid an unnecessary delay in the diagnosis of PML.

Taken together, these considerations drive an effort to revise the current PML diagnostic criteria and expanding them to include the less common JCV-associated entities mentioned above ([e88]).

PML immune reconstitution inflammatory syndrome

The term “PML immune reconstitution inflammatory syndrome” (PML-IRIS) was first introduced in HIV-positive individuals to describe the unmasking of PML with new-onset neurological symptoms following the commencement of cART or to describe clinical worsening following the initiation of cART in those already diagnosed with PML. PML-IRIS is reported in around 20% of PML cases in HIV-infected individuals [52]. PML-IRIS is also observed in natalizumab-associated PML, particularly after discontinuation of the causative DMT, and may be aggravated by accelerated removal of natalizumab using plasma exchange or immune adsorption [53]. PML-IRIS explains why a proportion of individuals with PML will become symptomatic after the treatment of an underlying condition has been initiated: a reconstituted cellular immune response against JCV-infected brain cells can result in overshooting inflammation, structural damage, and new symptoms, and may require specific anti-inflammatory therapy to prevent added harm.

As introduced above, individuals with PML can display gadolinium enhancement and perilesional edema on MRI, mainly observed at the lesion borders. This can be noted already at initial presentation or during the clinical course of PML, reflecting the amount of inflammatory activity or antiviral immune activity [40,42]. Histopathologically, this may correlate with pronounced lymphoid infiltrates in PML lesions and the surrounding white matter [48,54]. Marked evidence of inflammation on MRI (T1 gadolinum-enhancing lesions, swelling on T2/FLAIR), or histopathologically, in particular when perilesional edema is present, may raise the paraclinical suspicion of PML-IRIS (figure 3). Nevertheless, we suggest using the term PML-IRIS only when referring to the extreme with clinically relevant CNS inflammation, as a continuum of

inflammation is noted in PML, and a consensus definition of the term PML-IRIS based on MRI imaging or histopathological features has not (yet) been established.

PML prevention

Thus far, preventive measures for patients at increased risk of PML have exclusively been implemented for patients prior to or during natalizumab treatment. The risk mitigation strategy in place is based on the results of the Stratify-JCV^{TM} test, treatment history (prior use of classical immunosuppressants), and treatment duration, and may also include extended interval dosing in high-risk individuals who opt not to switch to alternative MS treatments available. The consequent application of such strategy, in particular the use of alternative therapy in JCV seropositives, is likely to reduce the overall PML risk in MS populations.

Additional promising biomarkers for PML prediction may include neurofilament light chain levels in blood, host genetic tests, monitoring of JCV genetic variants in various tissues, and prophylactic vaccine development based e.g. on JCV VP1 virus-like particles in populations at risk [34,55,56]. These strategies were mainly proposed for and studied in MS populations and did not exceed the experimental level. Considering PML being a rare disease, further validation and clinical implementation in particular outside the MS field may prove to be challenging.

Treatment of PML

Standard of care for PML

Restoring JCV-specific immunity remains key in clinical practice for treating PML. The approach depends on the underlying condition. Initiation of cART in HIV-infected individuals and the discontinuation of immunosuppressive therapy that caused PML are at the core of current management strategies [6]. A clinical study also demonstrated benefit on the PML survival rate following the optimization of a cART regimen in HIV positive individuals [57]. Added value of the use of plasma exchange or immune adsorption for enhanced drug clearance in natalizumab-associated PML is not confirmed [58] and needs to be weighed with the potential risk of triggering PML-IRIS on a case-by-case basis.

PML therapy studies listed on clinicaltrials.gov

As of January 2023, thirteen interventional studies had been listed on *clinicaltrials.gov* for PML or JCV-infection, excluding a study that tested an optimized cART protocol for HIV-PML (see above) and two studies that assessed therapies for PML-IRIS. Six of the thirteen studies listed are ongoing or remain in planning stage (table 2a).

Experimental direct antiviral therapy

Several antiviral treatments selected based on in-vitro evidence have been tested in small clinical trials (table 2a) or case series (table 2b), targeting different aspects of the JCV replication cycle, including DNA replication, retrograde transport, and viral entry. These treatments would be expected to work in all individuals affected by PML, regardless of the underlying disease and immune function. However, none of the drugs tested to date have shown beneficial effects on survival or neurological disability [2]. Among proposed antiviral compounds, mefloquine was the most recently proposed agent, studied in a phase II clinical PML trial that was terminated early [59].

Experimental treatments leveraging the immune response against JCV

The success of treatments that aim at increasing the host's JCV-directed immune response is anticipated to be variable depending on the underlying condition, genetic factors (such as HLA genes), and the phase of disease (early vs. advanced in PML).

Strategies used to restore JCV-specific T cell responses have included recombinant interleukin-2 (IL-2) and IL-7 that stimulate T cell growth, proliferation, and survival. While data thus far has been presented by anecdotal evidence from single cases or cases series (table 2b), a prospective pilot study investigating a long-acting recombinant IL-7 molecule is currently underway (table 2a).

Another approach described in clinical case reports and case series is the use of checkpoint inhibitors targeting programmed cell death protein 1 (PD1), thus far showing variable results. A study using pembrolizumab in HIV-associated PML listed on *clinicaltrials.gov*, with an estimated study completion date in 2021, provides no publicly available data yet (table 2a).

Probably most promising in clinical development is passive immunization using JCV- or BK polyomavirus (BKV)-specific T cells, each tested in several small retrospective cohort studies, and suggesting potential beneficial effects [60] (table 2b). A first open-label, pilot study using BKV-specific T cells [3], supported safety and feasibility. Definite demonstration of efficacy however remains to be investigated (table 2a).

Treatment for PML-IRIS

For the treatment of PML-IRIS, the standard of care is the cautious use of corticosteroids to minimize the damage resulting from a florid immune response, although higher and prolonged doses might interfere with effective viral clearance, possibly leading to negative outcomes. To date, standards for the type, dose or duration of corticosteroid treatment are lacking. Data on the added use of CCR5-blocking maraviroc did not convincingly

demonstrate a benefit, including one prospective study in HIV-associated PML patients (table 3).

Perspective and conclusions – toward PML becoming a treatable disease

Weighing the currently available possible treatment options, BKV- or JCV-specific T cells and enhancement of T cell responses using check-point inhibitors or recombinant cytokines appear the most promising experimental strategies and warrant testing in prospective clinical trials. Furthermore, recent advances in gene therapy, and the speed with which SARS-CoV-2 directed novel treatment options were advanced, create justified hope for the future development also of effective direct antivirals.

The development of PML-specific treatment, however, faces several methodological as well as ethical obstacles due to its rarity and severity as well as the heterogenicity of affected patients ([e89]):

- PML is a rare disease. Therefore, limited clinical data and few clinically established biomarkers are available. Recruiting large numbers of patients will also be challenging.
- PML being a relatively rapid and often fatal disease with no validated treatment so far, the recruitment, acceptability, and feasibility of complex study protocols might prove to be difficult.
- The heterogeneity of underlying diseases in PML individuals makes the selection of trial endpoints and interventional approaches challenging. The prognosis of PML depends on the underlying condition and the ability to reconstitute the immune system. Thus, large numbers of patients or more homogenous sub-cohorts are needed to determine which type of patient can benefit from which type of treatment strategy.
- Outcome measures for treatment response need to be sensitive, robust, and generally accepted. Ethical considerations may hinder use of robust clinical endpoints such as survival without offering rescue therapy to individuals that show deterioration, e.g., clinically or by MRI.

Considering the challenges mentioned above and aiming at a successful phase II/III clinical trial for PML, a study design using robust surrogate markers of response to therapy is needed. Possible surrogate biomarkers, still requiring validation, may include:

- Standardized MRI protocols and, e.g., the time to stable MRI [40]
- Fold change in JCV DNA load in CSF
- Robust measures of anti-JCV-directed humoral and T cell responses, including PML-type variant-specific responses, and CSF measures.

To improve PML diagnostics and identify surrogate markers of treatment response, at the German National Reference Center for Papilloma- and Polyomavirus, we currently work on developing more sensitive molecular assays for JCV DNA detection. These efforts comprise pre-analytic steps, including the enrichment of EV-associated JCV DNA, and the distinct quantification of different viral variants ([e90]). In addition, we work on high-throughput techniques, based on flow cytometry or microsphere hybridization assays, that might allow us to robustly study JCV viral-variant specific cellular and humoral immunity ([e91]). Efforts at the National Institute of Neurological Disorders and Stroke (NIH) include an ongoing natural history study of PML (NCT01730131) to identify robust surrogate outcomes, and the application of these to early-phase prospective interventional studies (table 2a). To raise awareness of this severe disease and to document the natural history of the disease as a basis for conducting therapy studies, the CurePML Registry was established in Hannover, Germany.

Sufficient funding and research efforts to overcome the remaining hurdles are warranted, considering PML is a devastating disease and a possible iatrogenic complication in a highly effective class of biologicals. As variable treatment responses can be expected, in particular for compounds that aim at increasing JCV-directed immunity of the host, the success of definitive trials that aim at demonstrating efficacy will critically depend on careful selection of study participants, based, e.g., on the underlying condition, the stage of the disease, and possibly also genetic factors. Nevertheless, considering the recent advances in understanding JCV biology, in biomarker development including imaging, blood, and CSF surrogates, as well as in immune therapeutics, we believe that PML may soon become a condition with more specific treatment options available.

List of abbreviations

AAN	American Academy of Neurology
AIDS	acquired immunodeficiency syndrome
BKV	BK Polyomavirus
cART	combined antiretroviral therapy
CNS	central nervous system
CSF	cerebrospinal fluid
DMT	disease-modifying treatments
DNA	desoxyribonucleic acid
EV	extracellular vesicle
GCN	granule cell neuronopathy
HIV	human immunodeficiency virus
IL	interleukin
IRIS	immune reconstitution inflammatory syndrome
JCV	JC Polyomavirus
MRI	magnetic resonance image
MS	multiple sclerosis
NCCR	non-coding control region
PCR	polymerase chain reaction
PD1	programmed cell death protein 1
PML	progressive multifocal leukoencephalopathy
VLP	virus-like particle
VP1	viral protein 1

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Figure legends:

Figure 1: Number of results per year on https://pubmed.ncbi.nlm.nih.gov/ (as of December 2022) of articles reporting on "progressive multifocal leukoencephalopathy". A first epoch of research can be noted between 1958-1980 (n=298), a second between 1981-2004 (n=1544), and a third starting in 2005 (n=2849).

Figure 2: Summary of findings in progressive multifocal leukoencephalopathy. According to current PML diagnostic criteria, for a definitive PML diagnosis, consistent clinical and imaging findings not better explained otherwise in combination with the detection of JCV DNA by PCR in CSF are needed (grey) [e79]. Alternatively, biopsy-based diagnosis (blue) requires the histopathologic triad coupled with a technique showing the presence of JCV in tissue [e80]. The figure was designed using biorender.

Figure 3: Magnetic resonance imaging findings in PML.

Kommentiert [AZ1]: Please provide a brief title for Figure 3.

A: axial fluid-attenuated inversion recovery (FLAIR), conventional T2-weighted, gadolinium-enhanced T1-weighted and diffusion-weighted imaging (including ADC mapping) typically used in clinical routine care for detection of PML. Multifocal leukocortical PML lesions involving both hemispheres with a more extensive lesion in the right parietal lobe involving the cortical grey matter, as well as the juxtacortical and deep white matter with multiple punctuate T2 lesions (arrows on the FLAIR and T2-weighted image). Some punctuate lesions show contrast enhancement. Focal areas show already a hypointense signal intensity in the T1-weighted sequence. Areas of high signal intensity on B1000 diffusion-weighted imaging with heterogenous ADC values indicate oligodendrocyte swelling suggestive of active viral replication (arrows).

(B): gadolinium-enhanced T1-weighted and axial FLAIR imaging before and 8 weeks following plasma exchange. Infratentorial PML lesions display more pronounced contrast enhancement on the gadolinium-enhanced T1-weighted image (arrows) and progressive hyperintense T2-FLAIR changes (arrows) partly due to perilesional edema and swelling following accelerated removal of natalizumab using plasma exchange (PLEX) in an individual who developed PML during therapy with natalizumab for MS. In a setting of a proposed reconstitution of the cellular immunity due to enhanced natalizumab drug clearance following PLEX, and a more pronounced cerebellar syndrome indicating clinical deterioration, these imaging findings fit with a diagnosis of PML-IRIS.

Figure 4: Typical PML lesion characteristics with demyelination. (A) LFB/PAS staining, myelin stained in blue is missing. Only some residual myelin sheaths are present on the left side of the picture. (B) HE staining, enlarged oligodendrocytic nuclei. (C) anti-VP1, within lesions, numerous virus-replicating glial cells are found. (D) anti-GFAP, bizarre astrocytes. (E), anti-CD3, typical for classic PML, only a few T cells are evident.

Table 1: Differences and similarities between HIV-associated and natalizumab-associated PML

	HIV-associated PML	Natalizumab-associated PML
Percentage of total PML cases	around 50%[6]	<5%*
Clinical presentation	depends on the locations involved, most common: cognitive and behavioral abnormalities, sensory and motor deficits, ataxia, aphasia, retro-chiasmal visual changes, seizures	like HIV-associated PML, but: -higher frequency of asymptomatic cases detected by magnetic resonance imaging screening
Magnetic resonance imaging	<u>in cART naïve individuals at initial clinical presentation ("classical PML"):</u> -T2 hyperintense lesions located in the juxtacortical and deep white matter; lesions do not respect the border between the white matter and the adjacent cortical grey matter (leukocortical lesions) -T1-isointense (early stage) or hypointense lesions (advanced stages) -on diffusion-weighted imaging (including apparent diffusion coefficient): diffusion restrictions particularly at the border of active lesions -T1 post gadolinium: minimal to no contrast enhancement <u>after cART initiation and during IRIS:</u> -active inflammation with contrast enhancement and perilesional edema	<u>at initial clinical presentation like HIV-associated PML but ("non-classical PML"):</u> - T1 post gadolinium: frequently (approximately in 30% of the patients) contrast enhancement particularly in the border of the main PML lesion - presence of punctuate T2 lesions in the vicinity of larger PML lesions ("punctuate pattern") occasionally showing contrast-enhancement <u>after natalizumab discontinuation (enhanced by PLEX/IA) particularly during IRIS:</u> - signs of severe inflammation with new or progressive contrast enhancement and perilesional edema, swelling
Cerebrospinal fluid findings	-routine parameter often normal or slightly elevated protein levels or mild pleocytosis -detection of JCV DNA by qPCR	-like HIV-associated PML, but: JCV-DNA copy numbers often low (<200 copies/mL) requiring repetitive testings
Histopathological findings	-histopathologic triad: demyelination, bizarre astrocytes, enlarged oligodendrocyte nuclei -tissue detection of JCPyV -few lymphoid cells and numerous virally infected cells	-like HIV-associated PML, but: more pronounced inflammation, fewer virally infected cells
Standard of care	-initiation of cART -cautious use of steroids for IRIS	-discontinuation of natalizumab treatment -(enhanced drug clearance by PLEX/IA) -cautious use of steroids for IRIS
Outcome eReferences1	Mean survival: 39.1%; 2006 and later (cART available): 52.5% (higher rates in smaller cohorts reported)	Mean survival: 90.5%

cART: combined antiretroviral therapy; IRIS: immune reconstitution inflammatory syndrome; PLEX: plasma exchange; IA: immune adsorption; DNA: deoxyribonucleic acid; qPCR: quantitative polymerase chain reaction; *decreasing numbers in recent years due to alternative multiple sclerosis therapy available and PML risk stratification using JCV serology

Kommentiert [AZ2]: When mentioning the eReferences, please provide the exact reference numbers. All 91 eReferences must be cited somewhere within the main text or figure/table legends.

Table 2: PML- and JCV-infection-directed clinical treatment studies registered with ClinicalTrials.gov and additional current treatment approaches recently published (2019-2023) as a case report, clinical study, or clinical trial

Kommentiert [AZ3]: Each table must be individually numbered without sub-tables. Please combine tables 2a and 2b into a single table or move one to an online-only supplement (eTable 1).

Study phase	Identifier	Principal Investigator/ Responsible Party	Drug	Rational	Primary Outcome	n participants	Status	Result	Publication*
Polyomavirus-specific T cells (PyVST) in studies registered with ClinicalTrials.gov									
pilot study	NCT02694783	I.C.M. Cortese, National Institute of Neurological Disorders and Stroke, USA	ex vivo generated PyVST from HLA-matched donor	enhancement of JCV-specific immune response	safety and feasibility	12	completed (2020)	no serious treatment-related adverse events	eReference[2]
phase I	NCT05101213	M, Daher, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA	glucocorticoid receptor knockout virus-specific T cells	enhancement of virus specific (ADV, BKV, CMV, JCV, SARS-CoV-2) immune responses	safety and feasibility	30	recruiting (2023)	N/A	N/A
phase I/II	NCT02048332	M. Grimley, Children's Hospital Medical Center, Cincinnati, OH, USA	donor-derived-VST following allogeneic stem cell transplantation	enhancement of virus specific (ADV, CMV, EBV, BKV, JCV) immune responses	successful VST production, rate of infusional toxicity, incidence of GVHD	450	recruiting (2023)	previous results: safe and effective therapy for the management of ADV infection in immunocompromised hosts	eReference[3]
phase II	NCT05541549	Cellevolve Bio Inc.	JCV-specific T Cell Therapy	enhancement of JCV-specific immune response	modified ranking scale score	60	not yet recruiting (2023)	N/A	N/A
phase II	NCT02479698	A, Olson, University of Texas, M.D. Anderson Cancer	BKV-specific T cell therapy	enhancement of BKV- and JCV-specific immune responses in	response, incidence of GVHD, incidence of	100	recruiting (2023)	previous results: safe and effective treatment for patients with BKV-associated hemorrhagic cystitis	eReference[4,5]

		Center, Houston, TX, USA		patients with malignancies	adverse events				
phase II	NCT02532452	M. Grimley, Children's Hospital Medical Center, Cincinnati, OH, USA	third party VSTs	enhancement of (ADV, CMV, EBV, BKV, JCV) immune responses	successful VST production, rate of infusional toxicity, incidence of GVHD	450	recruiting (2023)	safe and effective treatment for BKV and adenoviral-associated disease	eReference[3,6]
Immune response modulators in studies registered with ClinicalTrials.gov									
pilot study	NCT04781309	I.C.M. Cortese, National Institute of Neurological Disorders and Stroke, USA	NT-I7 (long-acting recombinant IL-7)	increase in lymphocyte numbers	change in absolute lymphocyte count	12	recruiting (2023)	N/A	N/A
pilot study	NCT00002270	University of Miami, Miami, FL, USA	interferon alfa-2b and zidovudine	immune reconstitution in HIV-related PML	N/A	N/A	completed (2005)	N/A	N/A
Immune checkpoint inhibitors in studies registered with ClinicalTrials.gov									
phase II	NCT04091932	B. Zhu, Zhejiang University, Zhejiang, China	pembrolizumab, PD-1 inhibitor	restoration of anti-JCV immunity	rate of non-progressors and recovers of AIDS patients with PML	10	estimated study completion date 2021	N/A	N/A
Antiviral treatments in studies registered with ClinicalTrials.gov									
phase I/II	NCT00746941	Biogen Inc., USA	mefloquine	in vitro evidence for JCV replication inhibition	JCV DNA load in CSF	37	terminated early (2010)	no beneficial effect	eReference[7, 8]
phase II	NCT00002395	SmithKline Beecham, UK	topotecan	in vitro evidence for JCV replication inhibition	safety and effectiveness (no details provided)	12	terminated early (2005)	inconclusive	eReference[9]

n.a.	NCT00000945	C.M. Marra, University of Washington School of Medicine, Seattle, WA, USA; D.E. Barker, Rush Medical College, Chicago, IL, USA	cidofovir probenecid	antiviral agent	safety and tolerability, neurological examination	24	completed (2001)	cidofovir did not improve neurological examination scores	eReference[10]
phase II	NCT00001048	C. Hall, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC, USA; J. Timpone, General Hospital, Washington, D.C., USA	cytarabine in combination with cART	restoration of the immune system in HIV-related PML	assessment of safety and efficacy	90	completed (1997)	no benefit of cytarabine	eReference[11]

Drug	**Number of treated patients**	**Reported outcome of PML**		**Publication**
		Improvement/ stabilization	**Death**	
Polyomavirus-specific T cells (PyVST) published as case reports, clinical studies, or clinical trials				
BKV-specific T-cells	3	3	0	eReference[12,13]
JCV-specific T-cells	10	6	4	eReference [14,15]
Immune response modulators published as case reports, clinical studies, or clinical trials				
Recombinant human IL-7	1	1	0	eReference[16]
IL-15 superagonist †	1	1	0	eReference[17]
Immune checkpoint inhibitors published as case reports, clinical studies, or clinical trials				
Atezolizumab	2	1	1	eReference [18,19]
Nivolumab	6	4	2	eReference[20–25]
Pembrolizumab ‡	27	18	9	eReference[26–42]

Atezolizumab, Nivolumab or Pembrolizumab, retrospective analysis of published and unpublished cases	79	41	38	eReference[43]
Antiviral treatments published as case reports, clinical studies, or clinical trials				
Cidofovir §/brincidofovir	5	1	4	eReference[44–47]
Cytarabine	1	1	0	eReference[48]
Ganciclovir	2	1	1	eReference[49]
Mefloquine	2	1	1	eReference[50, 51]
Mirtazapine	9	4	5	eReference[52–57]
Mefloquine plus mirtazapine II	15	12	3	eReference[58–71]

ADV, adenovirus; AIDS, acquired immunodeficiency syndrome; BKV, BK polyomavirus; cART, combined antiretroviral therapy; CCR5, C-C chemokine receptor type 5; CSF, cerebrospinal fluid; CMV, cytomegalovirus; DNA, desoxyribonucleic acid; EBV, Epstein-Barr virus; GVHD, graft-versus-host disease; HIV, human immunodeficiency virus; IRIS, immune reconstitution inflammatory syndrome; JCV, JC polyomavirus; MVC, maraviroc; N/A, not available; n.a., not applicable; PD-1, programmed cell death protein 1; PML, progressive multifocal leukoencephalopathy; PyVST, polyomavirus-specific T cells; VST, virus-specific T cells

† in combination with mefloquine and mirtazapine
‡ either alone or in combination with one or more of the following: IVIGs, IL-2, maraviroc
§ Two patients were also treated with mirtazapine and mirtazapine plus mefloquine, respectively.
II Four patients also received IVIGs.
IVIG, intravenous immune globulin

Table 3: Studies registered with ClinicalTrials.gov as treatment approaches of PML-IRIS, and recently published (2019-2023) case studies

Study phase	Identifier	Principal Investigator/ Study Chair/ Responsible Party	Drug	Rational	Primary Outcome	Number of Participants	Status	Result	Publication
phase IV	NCT01211665	Biogen Inc., USA	methylprednisolone prednisolone	To prevent and/or limit the deleterious immune responses in Natalizumab-associated PML-IRIS	Time course change in functional status based on Karnofsky Performance Status Index	3	Terminated due to lack of enrollment (2012)	N/A	N/A
Retrospective	NCT03969550	Fondation Ophtalmologique Adolphe de Rothschild, France	Maraviroc (CCR5 antagonist) as part of cART	To prevent and/or limit the deleterious immune responses in AIDS-related PML	Overall survival (OS), Time frame: 8 years	34	Completed (2015)	No clinically relevant benefit	eReference[72]

Drug	Number of treated patients	Reported outcome of PML		Publication
		Improvement/ stabilization	Death	
Steroids	4	4	0	eReference[73–76]
Maraviroc	2	2	0	eReference[77,78]