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MOLECULAR BASIS OF THE DEMYELINATING DISEASES

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Tesi di Dottorato di:
CAMILLA CARLONI
Matr. R08622

Direttore della scuola: Chiar.mo Prof. **Mario CLERICI**

Tutore: Chiar.mo Prof. **Pasquale FERRANTE**

Correlatore: Dott.ssa Serena **DELBUE**

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ABSTRACT

Multiple sclerosis (MS) is a multifocal demyelinating disease with progressive neurodegeneration caused by an autoimmune response to self-antigens in a genetically susceptible individual. Current first-line disease-modifying therapies for MS decrease the risk for exacerbations, the changes on magnetic resonance imaging (MRI), and the disability. In addition to the immunomodulatory therapies approved for MS, monoclonal antibodies (mAbs) have emerged as promising treatments to reduce the symptoms of MS. In particular, natalizumab (tysabri, Biogen Idec), binding the α4-integrin molecules on T-cells and preventing their entry into the brain and the consequent inflammation, reduces relapse frequency, delays the onset of disease progression and improves disease outcome in the relapsing remitting form (RR) of MS. However, it has been associated with Progressive Multifocal Leukoencephalopathy (PML), an opportunistic and often fatal demyelinating disease of the white matter of the human brain, caused by the human polyomavirus JC (JCV) lytic infection of oligodendrocytes.

By October 2012, a total of 298 cases of PML have been reported for 108,300 natalizumab-treated MS patients. Sixty-three out of 298 died, representing a mortality rate of 21,1%. Longer duration of natalizumab treatment, the use of immunosuppressants before the initiation of natalizumab therapy, and positive status with respect to anti-JCV antibodies, assessed with the use of a two-step anti-JC virus antibody assay (STRATIFY JCV, Focus Diagnostics) have been identified as risk factors for PML.

To better understand the molecular basis of demyelination, and to identify possible risk factors for the development of opportunistic infections and possible early prognostic markers of infectious complication during natalizumab treatment, an extensive longitudinal study has been performed, involving three different demyelinating diseases: MS, PML and Not Determined LeukoEncephalopathy (NDLE). The last one is a PML-like leukoencephalopathy, which occurs in HIV-1 positive subjects, but without evidences of JCV replication in the Central Nervous System (CNS). Biological fluids from 76 MS patients subjected to monthly natalizumab infusions or to other treatment, enrolled as controls, and from 28 PML and 22 NDLE patients have been collected at various stages of the diseases. JCV genome was searched and quantified by means of quantitative Real-Time PCR (Q-PCR), and molecularly characterized in order to monitor the viral presence and load, and to determine whether the viral organization may represent a risk factor for PML development.

The results obtained from these analyses showed that no JCV DNA has been amplified in the CSF from NDLE patients. On the contrary, JCV load and the molecular organization of the viral transcriptional control region (TCR) amplified in the CSF seem to represent the main markers of prognosis in HIV-1-related PML. An increased JCV urinary shedding was detected in the group of MS patients treated with natalizumab, compared to control group ($p < 0.05$). In particular, this increased JCV excretion was present up to the 24^{th} natalizumab infusion, whereas a decrease occurred after the 36^{th} infusion. Although JCV reactivation detected in urine from MS patients had a subclinical nature, it cannot be ignored and the constant molecular monitoring of urinary JCV DNA allows to identify patients who

harbor the virus and verifying whether viral replication/shedding in the urine is really influenced by the treatment with the drug. In addition, the anti-JCV antibodies test performed on natalizumab-treated MS patients showed a false negative rate of 7.9%, if compared with the results of JCV DNA test. Thus, testing JCV DNA, togheter with anti-JCV antibodies, represents a tool for PML risk stratification, also among patients who resulted negative at anti-JCV antibodies test, and may allow the identification of a specific, sensitive, reliable, reproducible and non-invasive biological marker of PML risk.

SOMMARIO

La sclerosi multipla (SM) è una patologia demielinizzante caratterizzata da neuro-degenerazione progressiva e causata da una risposta autoimmunitaria diretta contro auto-antigeni dell'organismo che si manifesta in soggetti geneticamente predisposti. Le terapie ad oggi disponibili riducono la severità e l'incidenza degli attacchi, così da prevenire le ricadute e prevenire o ritardare la progressione della malattia. Recentemente, in aggiunta alle terapie immunomodulanti basate sulla somministrazione di Interferone-β, Glatimer Acetato o Mitoxantrone, è stato introdotto il farmaco natalizumab (Tysabri, Biogen Idec), un anticorpo monoclonale umanizzato diretto contro l'integrina α4β1 (o VLA-4), che agisce impedendo l'adesione e l'extravasazione linfocitaria dal letto vascolare alla sede di infiammazione e, di conseguenza, previene i sintomi tipici della malattia e l'aggravamento della disabilità nei soggetti affetti dalla forma recidivante-remittente di SM. E' stata identificata, tuttavia, un'associazione tra trattamento con natalizumab e insorgenza di Leucoencefalopatia Multifocale Progressiva (PML), una patologia opportunistica e demielinizzante della sostanza bianca cerebrale, spesso fatale. Tale patologia è causata dalla riattivazione, in condizioni di immunodepressione, del polyomavirus umano JC (JCV) dal suo stato di latenza, con conseguente infezione litica e distruzione degli oligodendrociti.

Al mese di Ottobre 2012 sono stati riportati 298 casi di PML su 108,300 soggetti affetti da SM e trattati con natalizumab, con un tasso di mortalità del 21.1% (63/298 soggetti con PML sono deceduti). Tre fattori, singolarmente o in combinazione, sono stati associati a distinti livelli di rischio di sviluppare PML: positività agli anticorpi contro JCV, precedente uso di farmaci immunosoppressori e lunga durata del trattamento con natalizumab (> di 24 mesi).

Al fine di comprendere le basi molecolari della demielinizzazione e di identificare possibili fattori di rischio per l'insorgenza di patologie opportunistiche e possibili marker di diagnosi precoce di PML nei soggetti trattati con natalizumab, è stato condotto un ampio studio longitudinale, riguardante tre differenti patologie demielinizzanti: SM, PML e LeucoEncefalopatia Non Determinata (NDLE). Quest'ultima patologia, a eziologia non ancora definita, è caratterizzata da lesioni simili a quelle associate a PML, insorge in soggetti HIV-1 positivi ed è caratterizzata dall'assenza del genoma di JCV e di altri virus neurotropi a livello del liquido cerebro-spinale (CSF).

76 soggetti affetti da SM e trattati con natalizumab o con terapia convenzionale di prima linea (questi ultimi arruolati come controlli), 28 soggetti affetti da PML e 22 affetti da NDLE sono stati arruolati nello studio e campioni biologici di CSF, sangue, siero ed urine sono stati prelevati a diversi momenti della malattia. Il genoma di JCV è stato ricercato in tutti i campioni biologici raccolti tramite Real-Time PCR quantitativa (Q-PCR) e, quando amplificato, è stato sottoposto a caratterizzazione molecolare, al fine di determinare se l'organizzazione genomica virale potesse rappresentare un fattore di rischio per lo sviluppo di PML.

I risultati ottenuti hanno mostrato l'assenza del genoma di JCV nel CSF prelevato dai soggetti affetti da NDLE. Al contrario, la carica virale e l'organizzazione molecolare della regione di controllo trascrizionale virale (TCR) amplificata nel CSF dei soggetti affetti da PML sembrano rappresentare importanti fattori di prognosi.

Relativamente all'analisi sui soggetti affetti da SM, è emerso un aumento dell'escrezione urinaria di JCV nel gruppo dei pazienti trattati con natalizumab, rispetto al gruppo di controllo ($p<0.05$). In particolare, questo aumento si verifica fino alla 24ª infusione mensile di natalizumab, mentre, a partire dalla 36ª infusione, l'escrezione di JCV diminuisce fino a livelli pre-infusione.

Nonostante la riattivazione di JCV nelle urine si sia dimostrata subclinica, l'aumento della prevalenza del virus nei soggetti trattati con natalizumab non può essere trascurato e il monitoraggio costante dell'eventuale presenza di JCV nelle urine di tali pazienti potrebbe permettere l'identificazione dei soggetti infettati dal virus e di verificare se la replicazione virale e l'escrezione nelle urine possano essere influenzate dal trattamento con l'anticorpo monoclonale. Inoltre, confrontando i risultati del test anticorpale contro JCV, a cui tutti i pazienti sono routinariamente sottoposti, con i risultati ottenuti attraverso l'analisi molecolare, è stato possibile verificare come il primo test sia affetto da una percentuale di errore (falsi-negativi) del 7.9%. Risulta, quindi, fondamentale affiancare sempre la ricerca del genoma virale nei fluidi biologici del paziente nel tempo al test per la ricerca degli anticorpi anti-JCV, al fine di identificare i pazienti a rischio PML, anche tra coloro i quali hanno mostrato negatività al test anticorpale, e di trovare un marker biologico specifico, sensibile, riproducibile e facilmente determinabile in modo non invasivo di rischio di PML.

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1. Introduction

1.1 Demyelinating Diseases

Demyelinating diseases represent a heterogeneous group of diseases, which have in common the progressive loss of myelin from the nervous system. Since the myelin sheath of nerves is necessary for the conduction of neural impulses, this loss of myelin leads to an assortment of neurological symptoms that depend on the locations of the demyelinating lesions and may lead to disability and death. Important demyelinating diseases, affecting chiefly young adults, are immunomediated. The most common of these diseases is multiple sclerosis, an acquired disease of unknown etiology.
A second type of demyelinating disease is caused by viruses.
Finally, among hereditary neurodegenerative disorders, there are several categories of diseases due to the genetic lack of a particular metabolic enzyme causing a toxic build-up of chemical intermediates or a failure of myelin to form properly during development (dysmyelination).

1.1.1 Multiple Sclerosis

Over 160 years ago Charcot, Carswell, Cruveilhier and others first described clinical and pathological hallmarks of multiple sclerosis (MS), a multifocal chronic inflammatory demyelinating disease of the central nervous system (CNS) that leads to significant disability if left untreated.

1.1.1.1 Clinical Course

People with MS can typically experience one of four different disease courses, each of which might be mild, moderate, or severe (fig. 1).
Relapsing remitting (RR) MS is the most common type of MS, affecting around 85% of MS patients, and typically beginning in the second or third decade of life with a female predominance of approximately 2:1. It assumes that symptoms appear (a relapse), and then fade away, either partially or completely (remitting). A relapse is defined by the appearance of new symptoms, or the return of old symptoms, for a period of 24 hours or more, in the absence of a change in core body temperature or infections. In relapses, which can vary from mild to severe, symptoms usually come on over a short period of time, over hours or days, and they often stay for a number of weeks, usually four to six. The relapse, also known as attack or exacerbation, is followed by partial or complete recovery periods (remissions), during which no disease progression occurs [1,2].
Following an initial period of RR-MS, many people develop a secondary-progressive (SP) disease course characterized by a worsening of the disability, with or without occasional flare-ups, minor recoveries (remissions), or plateaus. Before the disease-modifying medications became available, approximately 50% of people with RR-MS developed this form of the disease within 10 years after the time of the diagnosis [3].
The primary progressive form (PP), usually diagnosed in people in their forties or fifties, is characterized by slowly worsening neurologic function from the beginning, with no distinct relapsing or remission. The rate of progression may vary over time, with occasional plateaus and temporary minor improvements [3].

The progressive relapsing (PR) is a relatively rare course of MS (5%), in which people experience steadily worsening disease from the beginning without remissions, but with clear attacks of worsening neurologic function along the way [3].
Finally, patients having a single bout of disease but not yet diagnosed with MS are referred to as clinically isolated syndromes (CIS), which is considered the first manifestation of the disease [4].

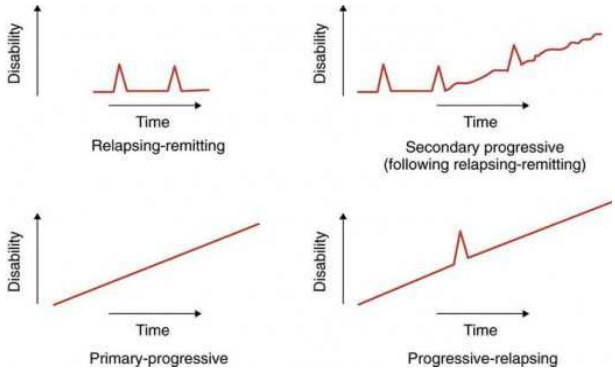


Fig. 1: the four different MS clinical courses.

1.1.1.2 Diagnosis

MS diagnosis is based on establish clinical and, when necessary, laboratory criteria. Magnetic resonance imaging (MRI) is particularly valuable in supporting the diagnosis. It shows the typical MS plaques in periventricular white matter, cerebellum, brain stem, spinal cord and also plaques as small as 3 to 4 mm. Moreover, using contras material, MRI identifies acute plaques and is useful in monitoring therapeutic efficacy. MS plaques are hypointense (black holes) on T1-weighted images [5].
Magnetic resonance spectroscopy (MRS) is helpul in demonstrating axonal injury. By measuring the concentration of N-acetyl aspartate (NAA), a neuron-specific marker, MRS monitors axonal dysfunction, which correlates with clinical disability. NAA levels are decreased in acute and chronic plaques. A temporary decrease in acute plaques is associated with reversible neurologic deficits. An increase in choline, a cellular membrane marker, is indicative of myelin breakdown [5].
Visual, somatosensory, and brainstem evoked potentials demonstrate a delay or a block in the conduction of nervous impulses and detected clinically silent plaques.
Cerebrospinal fluid (CSF) analysis often shows an increase in γ-globulin fraction and the presence of oligoclonal bands (OCB). OCB arise from the intrathecal production of clonal IgG, by clonally expanded B-cells also located in brain parenchyma, and are present in more than 95% of MS patients [5,6].

However, the definite diagnosis of MS is based on: (a) two or more episodes of neurologic deficits separated in time by at least 1 month, (b) two or more noncontiguous anatomic lesions on MRI, and (c) the absence of an alternative clinical diagnoses [5].

1.1.1.3 Epidemiological Features

The prevalence of MS varies considerably around the world. The prevalence is highest in northern Europe, southern Australia, and the middle part of North America [7]. Between 50 and 100 per 100,000 Caucasians (lower in the other ethnic groups) are afflicted with this disease, and women are afflicted more than men at a ratio of about 2:1 (fig. 2) [8]. In Italy, about 61,000 individuals are afflicted with MS, equal to 1 case per 1,050 inhabitant and 1,800 new diagnosed cases per year. The onset usually occurs between the ages of 15 and 50 with the greater incidence between 20 and 30 years [9].

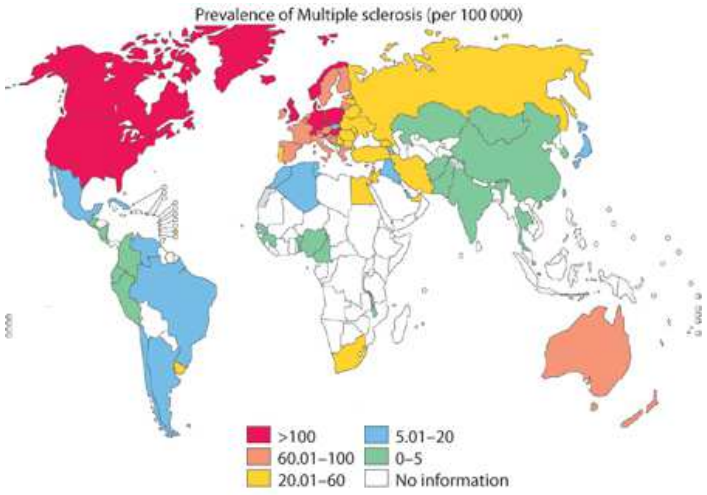


Fig. 2: prevalence of MS around the word for 100,000.

1.1.1.4 Etiology and risk factors

The etiology of MS remains elusive despite decades of research and a great interest in the disease. Three main hypotheses have been proposed: autoimmunity against the CNS, an infectious etiology, and a primary degenerative process affecting oligodendrocytes. These hypotheses are not mutually exclusive, as a primary viral infection can trigger autoimmunity or cause degeneration [10].

Evidence that genetic factors are at play in MS is unequivocal, but it is assumed that both the genetic background and environmental factors contribute to the disease manifestation. The high concordance rate of MS among monozygotic twins

(>25%), that is approximately six times the rate among dizygotic twins (5%), and the increased incidence in siblings of MS patients (2%) support some genetic factors [11].

During the last two decades, several investigative groups have collaborated to identify the individual genes that confer susceptibility to MS. The HLA class II extended haplotype HLA-DRB5*0101, HLA-DRB1*1501, HLA-DQA1*0102, and HLA-DQB1*0602 accounts for about 50% of the genetic risk of MS [12]. The whole genome scan effort has identified genes outside the HLA region as solid candidates for MS genetic risk. Single-nucleotide polyomophisms in the interleukin (IL)-2 and IL-7 receptors (IL-2, IL-7R) have been recently found to be associated with an increased risk for MS [13]. These allelic associations support an autoimmunity etiology in MS, as these alleles serve immune functions such as antigen presentation (HLA-DR), helper and regulatory T-cells functions (IL-2R), and memory T-cell (IL-7R). Some studies have reported immune deviation in MS, including cytokines imbalance in favor of a proinflammatory phenotype, deficiency in regulatory T-cells (Treg), and B-cells overactivity in the CNS reflected by the presence of increased IgG synthesis and oligoclonal bands in the CSF [10].

Potential environmental causes of MS, including behavioral and lifestyle as well as infectious agents, have been proposed in the etiology of MS.

A role for infectious agents (bacterial and viral) is supported by epidemiological studies. The equatorial region of the earth is a low risk area for MS and the risk increases as you move farther north or south from the equator. In this situation, infectious agents present in these areas may play a role. Indeed, migration studies suggested that, if migration from an area of high MS risk to an area of low MS risk or vice versa occurred prior to 15 years of age, then the migrant acquired the risk of the area to which he moved; whereas, if migration occurred after 15 years of age, then the migrant maintained the risk of area from which he moved [reviewed in 8,14].

Viruses have long been thought to play a role in the etiology and pathogenesis of MS. Studies in favor of viral involvement include the observation that isolated populations which had no reported cases of MS experienced epidemics of MS after contact with North Americans or Europeans [14,15] and, secondly, that CD8+ T-cells, cleaner of viral infections, were in the cellular infiltrate of active MS lesions. Moreover, exacerbation of MS can be correlated with viral infections, and, lastly, antibodies against various viruses and/or the viruses themselves have been directly isolated from MS patients [reviewed in 8].

Viruses that seem most associated with MS include rabies virus, herpes simplex virus (types 1 and 2), parainfluenza virus (type 1), measles virus, coronavirus, human T-cell lymphotrophic virus (type 1), and human herpes virus 6, all replicating extensively within the CNS [reviewed in 8].

The possible mechanisms by which viral infections could induce autoimmunity reactions that result in CNS inflammatory diseases are the molecular mimicry and the bystander activation. Molecular mimicry occurs when viral antigens have either sequential or structural similarities to self-antigens. So, viruses that encode molecular mimic could induce autoimmunity through either direct infection of the CNS or by peripheral infection. Instead, bystander activation is the nonspecific

activation of autoreactive cells due to inflammatory events caused by infection (fig. 3).
Among non-infectious risk factors, a protective effect of vitamin D in MS was recognized. This effect, in particular, is supported by the reduced risk associated with sun exposure and use of vitamin D supplements. Levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were significantly lower in RR-MS patients than in controls or patients with PP-MS [16]. Levels are also lower during relapse than during remission. These observations may be explained by the immunological effects of vitamin D, which include inhibition of myelin basic protein (MBP)-specific T-cell proliferation, enhanced differentiation of Tregs, and inhibition of IL-6 and IL-7 secreting cells [16].

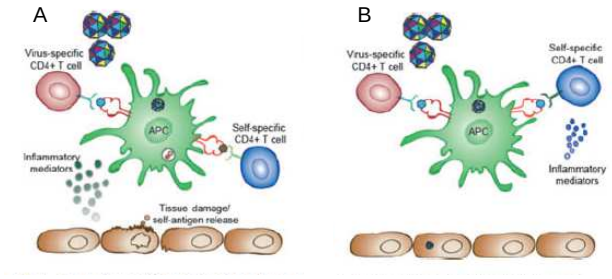


Fig. 3: mechanisms of viral induced autoimmunity. (A) bystander activation, (B) molecular mimicry.

1.1.1.5 Pathological features

The pathologic hallmark of MS consists of areas of focal demyelination, known as plaques, characterized by variable gliosis, inflammation and relative axonal preservation. The location, number, size, and shape of these lesions vary greatly among MS patients. They are disseminated throughout the CNS with a predilection for optic nerves, subpial spinal cord, brainstem, cerebellum, and periventricular white matter regions [17], but may also be found within grey matter, such as the cerebral cortex, thalamus, and basal ganglia.
Early/acute plaques are poorly demarcated, soft (reflecting edema and tissue lysis), slightly granular, and white or pinkish yellow. Long-standing chronic plaques are distinctly demarcated, firm, slightly retracted (reflecting tissue loss), translucent or gelatinous, and have a brownish discoloration.
MS lesions evolve differently during early versus chronic disease phases, and within each phase, different types and stages of demyelinating activity are evident. In the early stages of the disease, when patients present with clinical relapses and remissions, inflammatory demyelination leads to the formation of focal plaques. Instead, in later stages of the disease, chiefly in patients with SP or PP disease, additional pathology is seen, with a widespread demyelination in the cerebral and

cerebellar cortex as well as diffuse degenerative changes as in the white and in the grey matter [18].
Histologically, three basic processes characterize plaque formation: inflammation, myelin breakdown, and astrocytic fibrillary gliosis.
Inflammation: inflammation occurs with vasogenic edema and perivascular infiltration with lymphocytes, chiefly major-hostocompatibility-complex (MHC) class I restricted CD8+ T-cells. MCH class II CD4+ T-cells as well as B-cells or plasma cells are seen mainly in perivascular spaces and in the meninges, whereas their infiltration into the compact tissue of the CNS is sparse. Active tissue injury in MS lesions is associated with activated microglia and infiltrated macrophages [19]. In MS lesions, irrespective their stage of development, the CD8+ T-cell population always predominates and shows clonal expansion, and two waves of T-cell infiltrates are seen in MS lesions in the course of lesion maturation. In lesions with initial oligodendrocytes injury, demyelination and tissue injury massive pro-inflammatory microglia activation is present, associated with only a minor infiltration of T-cells, mainly MHC class I CD8+ cells. In addition, when myelin breakdown occurs and myelin debris are taken up by activated microglia and macrophages, a second wave of leukocytes infiltration, consisting predominantly of CD8+ cells but containing also CD4+ and B-cells, is seen [reviewed in 18].
Evidences suggest that the inflammatory response within the CNS may change with the progression of the disease. In particular, it decreases in severity with the age of the patients and disease duration, but active demyelination and neurodegeneration is invariably associated with inflammation, consisting of T-cells, B-cells, plasma cells and activated microglia and macrophages. At very late stage of the disease, inflammation may decrease to level seen in controls as the acute axonal injury [20].
Demyelination: the typical pathological feature of the sclerotic MS plaques is demyelination (fig. 4). Both the myelin sheath and the oligodendrocyte itself are destroyed within lesions, following attack by cells of the immune system that react with myelin-related epitopes, such as MBP. Immune attack involves both cellular immunity, with T-cells directed at myelin and oligodendrocytes and inciting phagocytosis by macrophages, and humoral immunity, with the secretion of anti-myelin antibodies from B-cells and subsequent fixation of complement and opsonization of the myelin sheath and the oligodendrocyte by macrophages. Multiple, well delineated, focal demyelinated lesions can be observed in the white matter of brains from MS patients, and these contain a high density of immunohistochemical markers for various immune cells, including different populations of T-cells, B-cells, macrophages and microglia, such as cytokines and chemokines. These immune cells are thought to have infiltrated the sclerotic plaque during the inflammatory episode that leads to demyelination.
Demyelinated plaques can also be observed in the cortical and subcortical grey matter.

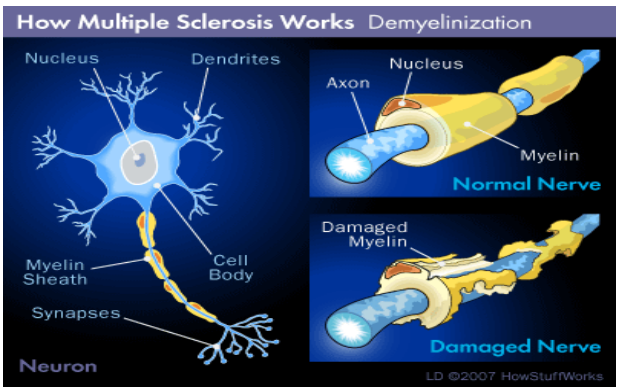


Fig. 4: demyelination: the pathologic hallmark in MS.

The biochemical and cellular mechanisms by which activated immune cells may destroy myelin and oligodendrocytes are multiple. The most common are presented below:

a. direct binding of T-cells to myelin epitopes can lead to activation of macrophages and to a subsequent attack of the myelin sheath leading to its phagocytosis;
b. release of cytotoxic cytokines or soluble toxic mediators such as nitric oxide from T-cells or microglia/macrophages can lead to destruction of myelin and myelin-producing cells;
c. antibodies directed against myelin epitopes released from infiltrating B-cells in the inflammatory lesion can bind to myelin, initiating fixation of complement, binding of macrophages, opsonization and phagocytosis of myelin and oligodendrocytes;
d. release of cytotoxic mediators from immune or glial cells, such as free radicals, which can cause oxidative stress, and glutamic acid, can create further tissue damage;
e. initial non-fatal damage to oligodendrocytes may initiate activation of an apoptotic cascade, perhaps by activation of death ligands or receptors such as Fas, FasL or Trail, that will result in delayed oligodendrocyte death. These apoptotic proteins can be activated by tumor necrosis factor (TNF), released from pro-inflammatory T-cells in MS lesions, and have been demonstrated to promote oligodendrocyte cell death *in vitro*;
f. injury to the myelin sheaths may render oligodendrocytes vulnerable to environmental toxins or viruses.

On the basis of patterns of staining for different immunological and cytological markers of cell injury and death, four different patterns of oligodendrocyte cell death can be distinguished in early acute MS plaques recovered from early disease [21].

Pattern I is characterized by sharp macrophage lesion borders, variable T-cell inflammation and T-cell-mediated autoimmune demyelination, and often extensive ongoing remyelination.
Pattern II is similar to pattern I, however, further distinguished by the precipitation of activated complement and IgG on degenerating myelin sheaths in active lesions, so it is a B-cell (antibody)-mediated autoimmune demyelination.
Pattern III active MS lesions demonstrate ill-defined macrophage borders with perivascular myelin sparing, where complement deposition is absent, with evidence of oligodendrocyte apoptosis and limited remyelination.
Lastly, pattern IV lesions contain extensive non-apoptotic oligodendrocyte degeneration in the peri-plaque white matter adjacent to the active lesion.
The B-cell-mediated pathology was the most frequently seen, in 53% of cases. These four different processes may occur to a different extent in different phases of disease; they are homogenous within a given patient but heterogeneous in different patient groups [reviewed in 22,23].
Astrocytic fibrillary gliosis: meanwhile, the astrocytes begin to proliferate and become round with homogenous eosinophilic cytoplasm and numerous fibrillary processes. As the plaques mature, the edema and the inflammation resolve, with a disappearance of macrophages. The astrocytes produce more fibers with consequently formation of a glial scar that fills the demyelinated plaque (astrocytic fibrillary gliosis) [5].
Important alterations affect also axons and oligodendrocyte. In axonal changes, a variable number of nerve fibers are damaged, some reversibly and some permanently, by the inflammatory process and by the loss of myelin sheats. During acute inflammatory demyelination, axons are probably damaged by the release of toxic inflammatory and nonspecific immune mediators in the lesion, such as proteases, cytokines, excitotoxins, and free radicals [24].
Regarding the oligodendrocytes, their number within the plaques may be reduced or they may be totally absent.
Within the plaques, the neurons may be unaffected, or they may show atrophy and some losses.
Remyelination: following acute inflammatory episodes, the resolution of the inflammatory processes is probably an important factor contributing to the neurological recovery often observed following clinical relapses. Complete remyelination give rise to the so-called “shadow plaques”, which are a demarcated area with reduced myelin density and thin myelin sheaths.
Although remyelination is generally incomplete and is characterized by thinner myelin sheaths and shorter internodal lengths than in the original myelin, older remyelinated plaques may show an almost normal myelin thickness, often indistinguishable from normal white matter [25].
Because of active demyelination, depletion of mature oligodendrocytes, and early remyelination occur simultaneously within some early MS lesions, the inflammatory microenvironment may have both a destructive and reparative functions. In fact, it appears that this remyelination is, at least in part, due to the activity of immune cells infiltrating the lesion. Immune cells in MS lesions can release anti-inflammatory cyrokines, such as IL-4, IL-10, and neurotrophic factors [26].

More than 40% of chronic MS lesions demonstrate evidence for remyelination [27], and in approximately 20% of MS patients, remyelination is so extensive that almost all plaques are shadow plaques [28].

1.1.1.6 Types of plaque

Staging of MS plaques: a classification scheme that attempts to distinguish demyelinating activity from inflammatory activity, and that might be present in the lesion even in the absence of ongoing active demyelination, relies on the structural profile and chemical composition of myelin degradation products within macrophages, in correlation to the expression of macrophage differentiation markers by immunocytochemistry [29].

Using this classification scheme, active MS lesions predominantly consist of reactive astrocytes intermixed with phagocytic macrophages and variable T-cells, both CD4+ and CD8+, B-cell, and plasma cell perivascular and parenchymal infiltrates. Early active MS lesions contain macrophages expressing myeloid-related protein (MRP14) and 27E10, both distinct markers of macrophage activation and differentiation. Since degradation of minor myelin proteins occurs rapidly, within one to three days, early active MS lesions contain myelin degradation products immunoreactive for minor myelin proteins including myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG), which disappear within 2 days, whereas late active lesions contain major myelin proteins including MBP and proteolipid protein (PLP), which are larger, more abundand, hydrophobic, and persist for 6-8 days. Inactive, demyelinated lesions are infiltrated by macrophages that lack degradation products of both minor and major myelin proteins. However, they contain empty vacuoles, the result of accumulation of neutral lipid components of myelin that cannot be digested and that accumulate and persist in macrophages. These macrophages can persist for up to six months in inactive lesions. Early remyelinating lesions are notable for the presence of clusters of short, thin, irregularly organized myelin sheaths, and are infiltrated by numerous lymphocytes and macrophages. Instead, late remyelinated lesions show few macrophages, astrocytic gliosis, and axons surrounded by newly formed thin myelin sheaths [23].

Type of MS plaques: a simple practical and diagnostic approach to classify MS lesions is based on the presence, the distribution, and the immunoreactivity of myelin-laden macrophages within the demyelinated lesions.

In the acute active demyelinated plaque, macrophages are distributed evenly throughout the lesion, and the majority contains both early and late myelin degradation products. These lesions are characterized by relative axonal preservation, variable loss of oligodendrocytes, and profound perivascular and parenchymal infiltrates composed mainly of T lymphocytes. Also, fewer B-cells and oligodendroglia are present in lesions.

Chronic active plaques are demyelinated lesions with relative axonal preservation and typically have sharply defined margins, with macrophages immunoreactive for both early and late myelin degradation products accumulating at the radially expanding plaque edge and diminishing in the inactive plaque center. Smoldering demyelinated plaques are characterized by an inactive center surrounded by a rim

of activated macrophages and microglia, few of which contain myelin degradation products.
Acute and chronic active plaques are more commonly found in fulminant or early MS, or in SP-MS patients still experiencing ongoing clinical exacerbations, whereas smoldering and inactive plaques predominate in patients with chronic progressive disease [30].

1.1.1.7 Pathogenesis

The hypothesis that MS is an autoimmune disease comes from the observation of similarities between MS and the animal model of the disease, experimental autoimmune encephalomyelitis (EAE). EAE is obtained by immunizing animals with myelin-derived proteins or peptides, such as PLP, MOG, MBP, and the disease is largely driven by myelin-specific CD4+ T-cells.
The inflammatory cascade associated to MS is tought to be mediated by a a coordinated attack by T-cells, monocytes, and B-cells against CNS tissue (fig. 5).

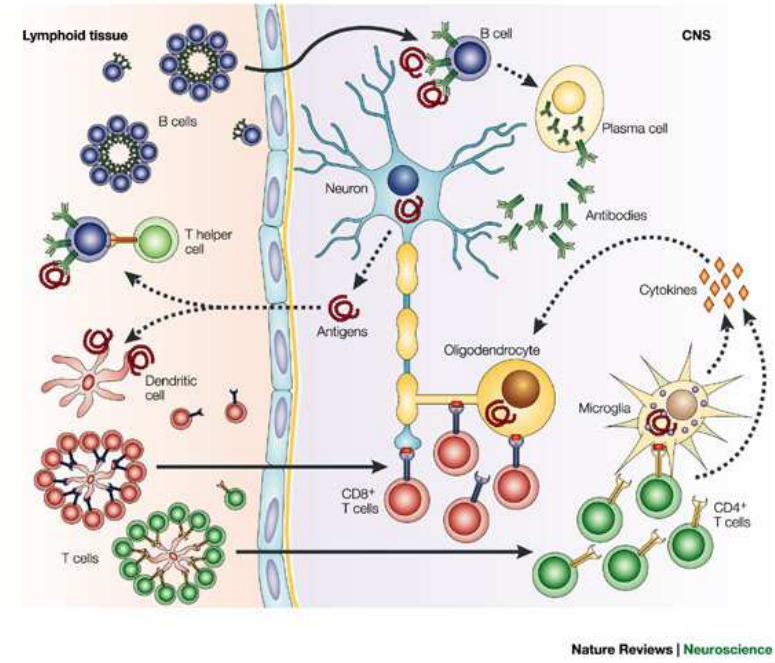


Fig. 5: pathogenesis of MS (from [31]).

Peripheral T-cell activation: myelin-specific autoreactive T-cells found in MS patients are in an “activated state”, which is associated with an up-regulation of adhesion molecules that make these cells more prone to interact with the Blood

Brain Barrier (BBB) and drive an inflammatory response directed against myelin antigens within the CNS. How these autoreactive T-cells from MS patients became activated in the periphery is still a matter of debate, and processes such as molecular mimicry, wherein T-cells generated against non-self epitopes, as viral or microbial agents, cross-react with self-myelin epitopes of similar sequence, or T-cell activation triggered by myelin antigens constitutively presented in cervical lymph nodes have been proposed as potential mechanisms [32-35].

T-cell migration into the CNS: After their activation in the periphery, autoreactive T-cells and activated monocytes must access and bind to the BBB. Initial steps in the migration through the BBB involve interaction between adhesion molecules expressed on the endothelial cells and immune cells. Two lymphocyte adhesion molecules and their binding partners have been implicated in the extravasation. The α4β1 integrin (VLA-4, very late activation antigen-4) and lymphocyte function-associated antigen (LFA-1), and their vascular and intercellular cell adhesion molecules, vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1), respectively, are all upregulated in vessels associated with MS lesions. Interestingly, there is a greater upregulation of VLA-4 and VCAM-1 in vessels of chronic lesions than in vessels of acute lesions, where LFA-1 and ICAM-1 were high in both, suggesting a possible switch to their preferential use in late stages of lesion development [36].

The crossing of the BBB by activated immune cells relies on degradation of the basement membrane underlying endothelial cells by metalloproteinases (MMPs), which are produced by a variety of cell types, including monocytes, macrophages, T-cells and endothelial cells. They are also made by cells of the CNS, such as microglia, astrocytes, and oligodendrocytes, and are used for remodeling and repair of cell matrix component. Chemokines are also involved in the migration of immune cells across the BBB, regulating the recruitment and the migration of these cells. Chemokines displayed at the endothelial lumen bind chemokine receptors expressed on circulating leukocytes and determine which leukocytes subsets will extravasate and enter the CNS [4].

Inside the CNS: the oligodendrocyte/myelin attack: activated T-cells specific for one or more self antigens migrate into the CNS, through the disruption of BBB. This process is followed by an amplification of the immune response after the recognition of target antigens on antigen-presenting cells (APC). In particular, within the CNS, myelin-specific CD4+ T-cells are reactivated in situ by myelin antigens presented in the context of HLA class II molecules in conjunction with other molecules on the surface of APC (as macrophages or microglia). This reactivation determines the release of pro-inflammatory cytokines and other soluble mediators that will further disrupt the BBB and stimulate chemotaxis, resulting in a second wave of inflammatory cells recruitment into the CNS. The existence of T-cells that are reactive to several self myelin and non-myelin antigens, including MBP, MAG, MOG, PLP, αβ-cristallin, phosphodiesterases, and S-100 protein, has been proposed [37-39]. Other amplification factors, including autoantibodies, are necessary to produce the demyelinated plaque [40,41].

Antibodies against antigens located on the surface of myelin sheath or oligodendrocyte can cause demyelination directly, through the activation of the

complement, leading to complement-mediated cytolisis. The antibodies may enter the CNS through the disruption of the BBB as a consequence of a T-cell initiated inflammatory response. Antibodies against both MOG and MBP can be found in patiens with MS and deposits of immunoglobulin and activated complement may be present in MS lesions, in which myelin is being degradated.
Other factors, which may help degrade myelin and damage oligodendrocytes, include activated macrophages and microglial cells, through the production of proinflammatory cytokines (such as TNFα and INFγ), the generation of reactive oxygen or nitrogen species, the production of excitatory amino acids, the activation of complement component, or the releasing of proteolytic and lipolytic enzyme. Other factors potentially toxic for oligodendroglial cells include soluble T-cell products, the interaction of Fas antigen with Fas ligand, cytotoxicity mediated by the interaction of CD8+ T-cells with class I MHC antigens on antigen-presenting cells, and persistent viral infections [reviewed in 1].

1.1.1.8 Cellular players of the immune response

CD4+ T-cells: following activation, naïve T-cells differentiate into different T-cell populations with various effector functions. Guided by cytokines produced by the dendritic cell (DC) that have been exposed to antigens, they differentiate into effector T helper cells (Th). T helper cells differentiation is initiated by signaling from DC to T-cells in the lymph node. Th1 cells, developed by INF-γ and IL-12 action, produce pro-inflammatory cytokines, such as INF-γ, that activate macrophages to kill intracellular pathogens, and TNF-α. IL-4 promotes Th2 subset differentiation, which secrete IL-4 and IL-5. IL-23, produced by macrophages and DC, and IL-1β promote the development of Th17 cells, that synthesize the pro-inflammatory cytokine IL-17A and IL-17F [42].
Th1 cells are believed to be further activated in the CNS by microglia, and subsequently they activate macrophages in the CNS to mediate myeline damage through Fc-R-mediated phagocytosis an the release of toxic mediators such as nitric oxide, proteases, free oxygen radicals, and TNF-α [43]. Th17 induces pro-inflammatory cytokines and chemokines, enhances DC maturation, and promotes neutrophil function [44]. The percentage of Th17 cells is increased in the blood of MS patients with active disease, and Th17 are found in the perivascular inflammatory cuff in MS brains with acute and chronic active lesions, but not in inactive lesions [45]. The Th17 to Th1 ratio appears to be a critical determinant of CNS inflammation, and high Th17 to Th1 ratios are associated with T-cell infiltration and inflammation in the brain parenchyma [46].
Regulatory T-cells (CD4+CD25+ Treg) comprise a small subset of CD4+ T-cells that have also been implicated in MS pathogenesis, although the number of Treg cells in peripheral blood and CSF appears to be similar between MS patients and control group.
CD8+ T-cells: Some evidences suggest that CD8+ T-cells have an important role in MS pathogenesis: 1) CD8+ T-cells are prominent in the inflammatory infiltrate in CNS lesions, and in some studies CD8+ T-cells outnumber CD4+ T-cells [47]; 2) infiltrating CD8+ T-cells are clonally expanded and may persist in the CSF for many years [48]; 3) CD8+ T-cells may promote CNS vascular permeability [49]; 4) adoptive transfer of activated myelin-specific CD8+ T-cells clones have been

shown to induce EAE, suggesting a key role for CD8+ T-cells in MS pathogenesis [50]; 5) axonal damage correlates with the number of CD8+ T lymphocytes infiltrating the lesion [51]; 6) CD8+ T-cells may participate in the axonal damage in MS by directly attacking neurons [52].

B-cells: B lymphocytes also play a key role in MS. This includes the presence of B-cells, plasma cells, Ig, and complement deposition in the MS lesion in the majority of patients. In addition, autoantibody and complement-mediated myelin damage have been demonstrated in affected tissues. Different observations suggest the importance of B-cells in MS: 1) B-cells isolated from CSF and MS brain lesions are clonally expanded; 2) there is a intrathecal production of oligoclonal Ig in the CSF from MS patients; 3) B-cells may directly participate in the demyelinating process by secreting pathogenic antibodies that target oligodendrocyte with or without the presence of the complement; 4) B-cell responses, such as proliferation, antigen-driven affinity maturation selection, and differentiation into antibody-producing plasma cells can be maintained locally within the CNS and may contribute to the pathogenic processes; 5) B-cells ablative therapy with the anti-CD20 monoclonal antibody rituximab, which depletes naïve and memory B-cells, was shown to reduce MS inflammatory brain lesions and clinical relapses [reviewed in 4].

1.1.1.9 Treatment

The treatment era for MS began in 1993, when the first disease-modifying therapy (DMT) was approved. This changed the focus of managing MS to the prevention of new disease activity. In fact, given the critical role of inflammation in MS, a key goal of MS therapy is the reduction or abolishment of related CNS inflammatory activity, with different therapies attempting to target different aspects of the aberrant immune response recognized to occur in MS. In addition to the known efficacy of anti-inflammatory therapies, the potential benefits of alternative strategies that may accomplish neuroprotection and/or remyelination in MS are recognized [53]. Currently there are eight different therapies for MS approved by the US Food and Drug Administration (FDA), and numerous new agents are being tested in clinical trials.

Interferon-β (IFN-β) and glatiramer acetate (GA), the longest approved MS DMTs, are relatively safe and commonly used as first-line agents. While benefits have been demonstrated across the range of RR form of MS, unfortunately neither IFN-β nor GA have shown convincing efficacy in PP-MS.

In 1993 IFN-β (with the commercial name Betaseron in the US, Betaferon in Europe) was the first DMT to be approved for the treatment of RR-MS, and additional IFN-β preparations have since become available for clinical use, IFN-β 1a (Avonex and Rebif, respectively), and IFN- β 1b (as Extavia). Depending on the formulation, IFN- β is administered either subcutaneously or intramuscularly, and either on alternate days, three times per week, or once weekly. Adverse events of IFN- β include injection-site reactions, flulike symptoms, leukopenia, deranged liver enzymes, and depression.

Although the exact mechanism of action is unknown, IFN-β drugs likely have multiple effects on the immune system [54]. The effects in the periphery include inhibition of antigen presentation, and at the BBB there is down-regulation of adhesion molecules and decreased production of matrix metalloproteinases. This

limits the T-cell activation and proliferation and the leukocyte migration across the BBB into the CNS. In addition, IFN-β determines apoptosis of autoreactive T-cells, modulation of cytokine production toward a more anti-inflammatory profile, and induction of regulatory T-cells [55]. IFN-β causes a shift from Th1 to Th2 cells, creating an anti-inflammatory milieu as an important mechanism in MS. However, more recently the effect of IFN-β on Th17 has been discovered to also be playing a role [56].

GA (Copaxone) is a random mixture of synthetic polypeptides composed of 4 amino acids, alanine, glutamic acid, lysine, and tyrosine. In the context of MS, a number of potential immunological mechanisms have been discussed for GA, including the generation of suppressor cells, induction of tolerance, expansion of regulatory T-cell populations, alterations of APC, and neuroprotective autoimmunity [57,58]. Common adverse effects of GA (a once-daily subcutaneous injection) include local injection-site reactions, postinjection systemic reactions, and lipodystrophy (with long-term therapy).

Other treatments, used as second line drugs, include mitoxantrone (Novantrone), fingolimod and oral cladribine. Mitoxantrone is a synthetic antineoplastic anthracenedione approved in 1987 as a treatment for acute myeloid leukemia and in 1996 for hormone-refractory prostate cancer [59]. It is a small molecule able to cross the BBB, where it interacts with the cells in the CNS, to inhibit DNA replication and DNA-dependent RNA synthesis as well as topoisomerase II ability to repair DNA. This ultimately leads to apoptosis of both proliferating and nonproliferating cells. Mitoxantrone has broad effects in MS resulting in suppression of T-cell, B-cell, and macrophage proliferation, decreased secretion of proinflammatory cytokines (IFN-γ, TNF, IL-2); enhanced T-cell-suppressor function, inhibition of B-cell function and antibody production, and inhibition of macrophage-medicated myelin degradation. Mitoxantrone is given by intravenous infusion, as it is poorly absorbed orally. The efficacy of mitoxantrone in MS has been evaluated in four randomised controlled clinical trials. It was approved by the FDA in 2000 for worsening forms of MS including worsening RR-MS, SP-MS, and PP-MS. However, its use is limited by safety concerns. It is generally well-tolerated, although associated with nausea, urinary tract infections, menstrual disorders, amenorrhea, and mild alopecia when compared with placebo.

Oral fingolimod was approved for the treatment of MS by the FDA and the European Medicines Agency (EMA) in 2010. Fingolimod is licensed for use as a first-line agent in the United States, but only as a second-line agent for active MS in Europe. Fingolimod, a synthetic analogue of the immunosoppressive fungal metabolite myriocin, is a sphingosin-1-phosphate (S1P) receptor modulator for once daily oral administration. In vivo, it is phosphorylated to yield the biologically active agent that binds to S1P receptors, modulating MS pathology, potentially acting both on the peripheral immune system and at central levels [60]. Fingolimod binding to S1P receptors on lymphocytes prevents their egress from lymph nodes, resulting in a dose-related reduction in the number of circulating lymphocytes and a reduced infiltration of autoaggressive lymphocytes into the CNS. Preclinical findings suggest that fingolimod may also promote neuroprotective and reparative

processes within the CNS through modulation of S1P receptors on neural cells. For these reasons, fingolimod is currently being investigated in PP-MS.
Cladribine, an antilymphocytic drug, is an analog of the purine nucleoside adenosine [61,62], and induces apoptosis and depletion of both proliferating and quiescent lymphocytes, sustained depletion of CD4+ T-cells, less pronounced dose-dependent reductions in CD8+ T-cells, and smaller dose-dependent reductions in CD19+ and CD16+/CD56+ lymphocytes. As expected for a drug with preferentially lymphotoxic effects, myelosuppression is the major dose-limiting effect, and leukopenia, neutropenia and thrombocytopenia may occur. Cadribine was recently approved only in Australia and Russia as a treatment of RR-MS.

1.1.1.9.1 New biological drugs

As our understanding of the pathogenesis of MS has evolved, therapeutic strategies have been developed to target specific cells and processes that contribute to the myelin and axon damage characteristic of MS. In addition to the immunomodulatory therapies that have been approved for MS, monoclonal antibodies have emerged as promising treatments to reduce the symptoms of MS [reviewed in 63].
Monoclonal antibodies (mAbs) are a relatively new class of biologic agents that allow targeting of specific proteins on cell population and have shown promising in the treatment of a number of autoimmune diseases. Because they are designed to target specific antigens, mAbs may have more selective effects than drugs that potentially interact with multiple targets, although they still may have widespread effects on the immune system. By targeting specific antigens, mAbs potentially could provide improved efficacy compared with systemic immune-modulating therapies. MAbs approved or under investigation for MS include natalizumab (Tysabri), alemtuzumab (CamPath), rituximab (Rituxan) and daclizumab (Zenapax).
The pathology of MS is thought to involve a cascade of immunologic processes involving T-cells, B-cells, macrophages, monocytes, DC, and an array of proinflammatory mediators and cytokines. Therefore, the modulation of these cells and their pathologic actions has been seen as a likely therapeutic target of the mAbs.

1.1.1.9.2 Natalizumab

Natalizumab (Tysabri [Biogen Idec, Zug, Switzerland and Elan, Athlone, Ireland]) is a humanized mAb that contains 90% human sequences and only 10% foreign sequences, represented by the amino acids from the mouse IgG in the variable regions that are needed to make the antigen binding site.
It binds to the α4-integrin, a component of VLA-4 present on several leukocytes subtypes, in particular on T-cells. Binding of the antibody block the interaction with the ligand VCAM-1 on the surface of endothelial cells at the level of BBB, preventing the transmigration of leukocytes across the endothelium into inflamed parenchymal tissue [64] (fig. 6). In addition, some reports evidence that a long-term natalizumab therapy decreases the number of APC and the expression of MHC class II in cerebral perivascular spaces, which in turn may affect the reactivation of T effector cells in the CNS.

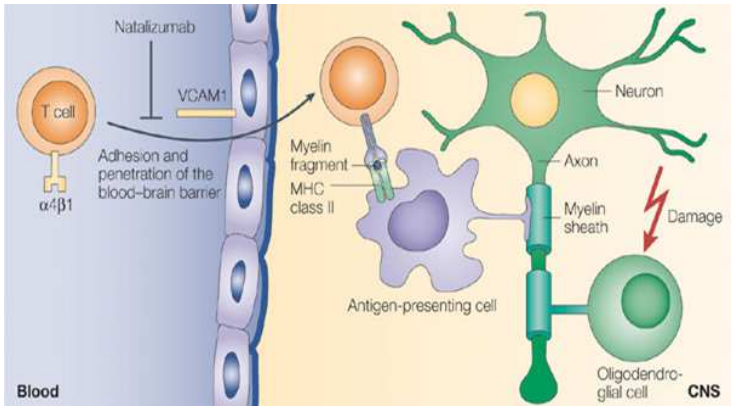


Fig. 6: natalizumab: mode of action.

Alhough natalizumab has a biological half-life of 11 days, it is possible to detect in the CSF of natalizumab-trated patients, six months after the cessation of the therapy, few CD4+ T-cells, CD8+ T-cells, CD19+ B-cells, and CD138+ plasma cells, demonstring that natalizumab therapy may significantly impair immunosyrveillance in the brain and spinal cord. In addition, peripheral blood CD4+/ CD8+ ratio decreases significantly with increasing number of natalizumab doses, but remaines within normal limits. This may suggest that the differential effect of α4 integrin on cell numbers of CD4+ T-cells and CD8+ T-cells in peripheral blood may be a consequence of their activation and differentiation in secondary lymphoid organs [65].
Natalizumab was approved for the treatment of patients with MS who have the active RR form of the disease on the basis of its target mode of action and its positive effects on various clinical and MRI outcomes in the placebo-controlled clinical studies AFFIRM (Natalizumab Safety and Efficacy in Relapsing-Remitting MS), SENTINEL (Safety and Efficacy of Natalizumab in combination with Avonex [INFβ-1A] in Patients with Relapsing-Remitting MS), and GLANCE (Glatimer Acetate and Natalizumab Combination Evaluation) [reviewed in 66]. In particular, in the AFFIRM trial natalizumab was applied as a monotherapy in 942 MS patients who had not received any immunotherapy in the preceding 6 months. 96% of subjects in the treatment arm were devoid of new gadolinium-enhancing lesions compared to 68% in the placebo group. Relapse frequency was reduced by approximately two thirds over year one and two and natalizumab also significantly delayed disease progression [67]. The SENTINEL study tested the combination of natalizumab with IFNβ-1a once-weekly against IFNβ-1a alone. Individuals receiving combination therapy had a reduced relapse rate by 54% over IFNβ-1a alone and significantly fewer MRI lesions [68].
Natalizumab is administered intravenously every 4 weeks and, in general, the treatment is well tolerated with infections, headache, fatigue, urinary tract

infections, arthralgia, and depression as the most common adverse effects in the monotherapy [67]. Based on the positive interim analysis of phase III AFFIRM and SENTINEL trials, natalizumab was approved by the FDA for the treatment of relapsing forms of MS in November 2004.

Commercial and clinical trial dosing of natalizumab was suspended voluntarily in February, 2005, when two patients in the combination trial of natalizumab and IFNβ-1a developed Progressive Multifocal Leukoencephalopathy (PML) [69,70]. In addition, a third case of PML was diagnosed in a patient receiving natalizumab for the treatment of Crohn's disease [71].

The incidence of PML was estimated to be approximately 1 case per 1000 MS patients treated with natalizumab. However, the drug was reintroduced in the USA and approved in the European Union in June, 2006, after no additional cases of PML were identified in previously treated individual. The FDA approved an application for resumed marketing of natalizumab as a monotherapy in patients with relapsing forms of MS who have not responded adequately to, or cannot tolerate other treatment. To minimize the risk of further serious adverse events and to continue the assessment of natalizumab safety profile in clinical practice, a special restricted distribution program and a risk management plan were developed, including the TOUCH (Tysabri Outreach Unified Commitment to Health) prescribing program, mandatory in the USA, and the TYGRIS (Tysabri Global Observation Program in Safety). In Europe, natalizumab has been approved by the EMEA as monotherapy for patients with high disease activity despite sufficient treatment with IFNβ as well as for patients with initially high disease activity.

As of October 3, 2012 there have been 298 confirmed cases of PML among 108,300 natalizumab-treated MS patients, with 63 deaths and a mortality rate of 21.1%.

As natalizumab and other biological drugs has a suppressive action on the immune system, this effect may increase the risk for opportunistic infections. Although the overall incidence of infections was low, in the monotherapy pivotal trial, certain types of infections were more common in patients treated with natalizumab than in placebo-treated patients (eg, pneumonias, urinary tract infections, gastroenteritis, vaginal infections, tooth infections, tonsillitis, and herpes infection).

1.1.1.9.3 Other monoclonal antibody therapies

Alemtuzumab (previously called Campath-1H; Campath in the US [Genzyme, Cambridge, MA, USA and Bayer Health Care Pharmaceuticals] and MabCampath in Europe [Genzyme and Bayer Schering Pharma AG, Berlin, Germany]) is a humanized mAb directed against the CD52 antigen, which is present on the cell surface of T and B lymphocytes, monocytes, macrophages and eosinophils but not stem cells. It might deplete these cells through complement mediated lysis, antibody-dependenT-cell toxicity and induction of apoptosis, and therapy with this mAb results in a long-term immunosuppression. Alemtuzumab is already licensed for the treatment of refractory chronic lymphocytic leukemia and is under investigation in the treatment of MS [72]. The results of the alemtuzumab studies in MS report generally consistent safety profiles, with the most prominent adverse effect being the development of Graves' disease, an autoimmune hyperthyroidism. In addition, the profound lymphopenia associated with alemtuzumab administration

results in an increased incidence of infections (which have included cases of measles, spirochetal gingivitis, herpes zoster, varicella zoster, recurrent oral aphthous ulcers, and pyogenic granuloma) [73].

Daclizumab (Facet Biotech, Redwood City, CA, USA and Biogen Idec) is an IL-2 receptor antagonist that specifically targets the binding site on the IL-2 receptor α chain, CD25. Because CD25 is present at low levels on resting T-cells, but exists at particularly high levels on activated T-cells, it is thought that selective targeting of CD25 will correlate with selective blocking of activated T-cells. Further, because T-lymphocyte activation is central to the inflammation characteristic of MS lesions, this blockade theoretically would reduce the inflammation in the brain associated with MS lesions [74-76].

Daclizumab is approved by the FDA for the treatment of renal transplant rejection and has been tested as a therapy for cardiac allograft rejection [77]. Daclizumab has been studied for the treatment of MS as adjunctive therapy in patients failing INF treatment.

In the safety analysis, was found an increase in the number of infections during the daclizumab treatment phase, although they were all mild urinary and upper respiratory tract infections common in MS. Other analysis have seldom detected paresthesias, mild leukopenia without lymphopenia, mild anemia, rash, and transient low-level increase of liver enzymes [74,75].

Rituximab (Rituxan in the US [Genentech, San Francisco, CA, USA and Biogen Idec, Weston, MA, USA] and MabThera in the rest of the world [Roche, Basel, Switzerland]) is a chimeric mAb (murine variable regions fused onto human constant regions) against the CD20 antigen, which is present on pre-B-cells and B-cells, but not on antibody-producing plasma cells or stem cells in the bone marrow. Administration of rituximab results in B-cell depletion with preservation of antibody production by plasma cells. B-cells number is restored in the peripheral circulation in approximately 3 to 12 months after treatment.

Rituximab is approved by the FDA for treatment of B-cell lymphomas [78,79] and is under investigation as a therapy for a number of autoimmune diseases. There are several completed or ongoing investigations of rituximab as a treatment for MS [reviewed in 80]. Reduction in the number of B-cells and T-cells from the circulation and the CSF has been observed following rituximab treatment [reviewed in 80]. A recently published study has demonstrated the potential of rituximab as a very effective therapy for MS, with a marked reduction in new enhancing lesions maintained for 48 weeks. Clinically, these patients also had a significant reduction in relapses sustained over this time period [81].

Adverse effects of therapy, observed with the initial infusion and substantially less following the second mAb infusion, included chills, headaches, nausea, fever, fatigue, throat irritation, and pharyngeal pain. Potential rituximab-associated conditions include serum sickness and PML, which have been observed during therapy of other conditions, including systemic lupus erythematosus (SLE), but not in MS patients [82].

Since PP-MS has been refractory to most immunotherapies thus far, proving that rituximab can be effective for PP-MS would be a major advancement in MS therapeutics.

1.1.2 Progressive Multifocal leukoencephalopathy

PML is a rare and often fatal demyelinating disease of the CNS that was first reported in 1958 by Astrom and colleagues [83], following the examination of brain tissue from two cases of chronic lymphocytic leukemia and one case of Hodgkin lymphoma. PML occurs as a devastating neurological opportunistic infection in the contest of severe impairment of the immune system, such as Acquired ImmunoDeficiency Syndrome (AIDS) or treatment with immunomodulatory compounds, mainly a number of mAb and it is caused by the Polyomavirus JC [84,85].

1.1.2.1 Epidemiology of PML

Prior to the era of Human Immunodeficiency virus 1 (HIV-1), PML remained a relatively rare disease seen in few immunosuppressed patients, including individuals with hematological malignancies, organ transplant recipients and people with chronic inflammatory conditions, with an estimated incidence of 0.07% [86].

However, PML prevalence dramatically increased during the AIDS epidemic, where up to 5% of AIDS patients developed the disease. Mortality related to PML has also increased from 1.5 per ten million persons in the pre HIV era to 6.1 deaths per ten million persons in the post HIV era [87].

Since 1996 highly active antiretroviral therapy (HAART) became the standard practice for HIV treatment [88]). It has reduced the incidence of and mortality due to PML in patients infected with HIV-1. Before the use of combined antiretroviral therapy, PML affected 3–7% of HIV-1 positive patients and was the cause of up to 18% of fatal CNS diseases. The incidence (rate of new cases) has decreased substantially with combined antiretroviral therapy, although not to the same extent as that of other opportunistic infections of the CNS [reviewed in 89]. In pre-HAART era the incidence of PML varied from 0.3% to 8%, but the wide use of antiretroviral treatments led to a significant reduction from 0.7 cases per 100 patient-years to 0.7 cases per 1000/ person-years at risk [reviewed in 90].

Prior to HAART, AIDS-associated PML (AIDS-PML) was typically fatal within 6 months from the diagnosis, but, with the use of antiretroviral therapy, survival among patients in this PML population has begun to improve, changing from 0-30% 1 year after diagnosis to 38-62% since the introduction of HAART [88,91].

However, HIV infection is still the most commom immunodeficiency setting for PML, accounting for about 80% of cases, followed by hematologic malignancies (~8%), solid cancer (~3%), organ transplants and autoimmune disease treated with immunomodulators [92].

1.1.2.2 Neuropathology and image findings

The central pathologic feature of PML is the infection of the oligodendrocytes with the polyomavirus JC (JCV), which leads to lysis of the cell. The infection spreads to surrounding oligodendrocytes and results in focal destruction of myelin; microscopic foci of myelin breakdown coalesce to form larger lesions, primarily in subcortical white matter and then evolving towards the deep periventricular regions, involving also thalamus and basal ganglia. Lesions are most commonly present in the cerebral hemispheres, but may also affect the cerebellum and the brain stem [93].

PML may affect the gray matter, usually in association with white matter involvement [83,94]. The infected oligodendrocytes have inclusions of viral particles in the nuclei, which give the appearance of inclusion bodies and loss of chromatin structure upon examination by light microscopy. Oligodendrocytes are diminished in number and are principally located at the lesion's periphery. The size of the nucleus may also enlarged by as much as 2- to 3-fold. The multifocal nature of the lesions suggests a hematogenous spread of the virus to the brain.

To a lesser extent, astrocytes are also infected with JCV. These cells appear enlarged, with hyperchromatic, deformed and "bizarre" nuclei containing JCV particles; the nuclei appear irregular, lobulated and premitotic, but do not become neoplastic (fig. 7).

Although neurons themselves are rarely productively infected by JCV, demyelination leads to axonal dysfunction, and the demyelinated axon is susceptible to injury by cellular products released by the glial cells. Axonal injury can result in a retrograde loss of the neuronal cell body. Loss of neurons is likely permanent.

Conventional MRI shows an extensive involvement of subcortical white matter, with multifocal, bilateral, asymmetrical lesions in early stages of the disease, that appear hyperintense in T2-weighted sequences and hypointense in T1-weighted sequences (fig. 7).

Invading macrophages are commonly seen in the centers of lesions. They act as scavengers and are actively involved in removal of myelin debris in the sites of lesions. Macrophages and microglia are not infected by JCV.

Although there are no fundamental differences between the pathology of PML in patients with and without AIDS, several subtle differences have been identified. In AIDS-PML patients, there can be massive necrotic lesions with infiltration by HIV-infected macrophages [95], and grey matter and infratentorial lesions are also more common than in non-AIDS-PML patients.

However, the location of the lesions is generally similar in AIDS- and non-AIDS-PML patients [97,98].

Lymphocytes are not typically seen in PML lesions except if there is restoration of the immune system leading to an Immune Recinstitution Inflammatory Syndrome (IRIS). Under these circumstances, CD8+ T-cells are the predominanT-cell type and are present in perivascular regions at sites distant from areas with JC virus infection as well as in focal collection in the parenchyma in proximity to JCV-infected cells [99,100]. The presence of cyto-toxic T-cells against JCV is considered to be a good prognostic sign. It is postulated that the rapid restoration of the immune system may lead to an expansion of activated T-cells, which may contribute to the massive inflammatory response seen in patients with IRIS and can result in significant morbidity and mortality.

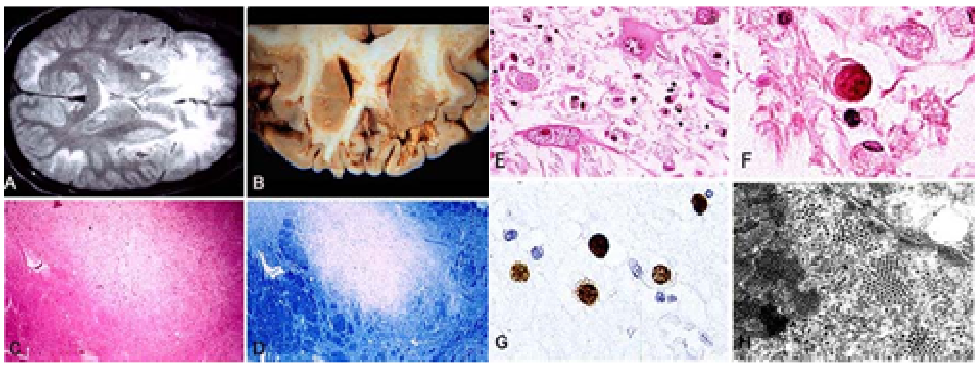


Fig. 7: histopathological features of PML: (A) MRI of the brain showing the characteristic hyper-intense areas located in the sub-cortical white matter; (B) extensive lytic lesions in the sub-cortical white matter; (C) extensive areas of myelin loss; (D) demyelinated areas highlighted with a special stain for myelin; (E) giant reactive astrocytes with bizarre atypical nuclei; (F) enlarged oligodendrocytes harboring intranuclear eosinophilic inclusion bodies; (G) immunohistochemistry for the JCV capsid protein VP-1 shows robust reactivity in the nuclei of infected oligodendrocytes, demonstrating productive infection; (H) the intranuclear inclusion bodies in oligodendrocytes are composed by numerous icosahedral viral particles as demonstrated by electron microscopy (from [96]).

1.1.2.3 Clinical features

Signs and symptoms: PML has a tumultuous course and a poor prognosis within few months [84,101]. The introduction of HAART has modified the HIV-related CNS disease scenario, and new form with different clinical presentation and better prognosis have been described [102]. The classical form of the disease have a multisymptomatic onset, with symptoms varying on the basis of the location and the size of lesions. However, the most frequent clinical presentation is characterized by motor deficits, altered consciousness, gait ataxia, and visual symptoms. Atypical presentations include pure cerebellar syndrome, reflecting a productive infection of granule cell neurons, meningitis, meningoencephalitis, progressive myoclonic ataxia, and muscle wasting associated to extrapyramidal signs [90,101,103].

Diagnosting testing: diagnosis can be divided into three stages: clinical suspicion, radiological identification, and confirmation of cause by CSF or tissue analysis [104]. The first of these stages relies on the character and development of focal neurological symptoms and signs over time and disease susceptibility.

The second stage in diagnosis involves the detection and characterization of brain lesions by MRI, which shows characteristic white-matter lesions in brain areas associated with the clinical deficits. Because the lesions involve demyelination, they are usually hyperintense on T2-weighted and FLAIR (Fluid-Attenuated Inversion Recovery) MRI sequences, but hypointense on T1-weighted sequences,

indicating white matter destruction. The hypointense lesions help distinguish PML from other pathologies, primarily HIV-1 encephalopathy, with more diffuse central white-matter changes that are not detected on T1 sequences [89].
Definitive diagnosis can only be made when PML is confirmed by histopathology, which must evidence a consistent neuropathology in the brain (biopsy or autopsy) with JCV DNA or protein detected by in situ techniques.
The CSF-confirmed PML has a lower degree of diagnostic given the variation in sensitivity and specificity of Polymerase Chain Reaction (PCR) assays in different laboratories. The detection of JCV DNA in CSF by PCR had a sensitivity of 72-92% in the pre-HAART era, but fell to 58% after the introduction of HAART [105,106]. This is probably related to the better immune control of the virus and the higher CSF clearance of the virus by immune cells.
The occurrence of PML in patients treated with novel immunomodulant therapies has stimulated the search for biological markers that are more easily accessible, reliable and detectable than CSF for the early phase of the disease [85,107,108].
So far, no blood biomarkers of JCV activity have been identified.

1.1.2.4 Therapeutic options

Classical treatment of PML: the prognosis of PML is generally poor. In the pre-AIDS era and before antiretroviral drugs were available, death was nearly universal, with an average survival of 9 months in non-HIV patients and 2 to 4 months in patients with HIV infection [103].
At present, there is no specific antiviral drug against JC virus, but a number of treatment options have been used for PML, largely without success. The main approach to treatment of HIV-1-related PML involves combined antiretroviral therapy with the objective of reversing the immunological defect that interferes with the normal host response to JCV. This is therefore an indirect approach to treatment, but it is the only one that has proven effective.
In patients with both PML and HIV-1, combination antiretroviral therapy stops progression of leukoencephalopathy in about half of those who receive it [109]. Unfortunately, neurological deficits frequently persist because of irreparable loss of brain tissue, and only a few patients functionally improve.
Other treatment options used for PML treatment, but without success, have targeted various point in viral cycle such as virus entry and replication, and includes cidofovir (CDV), cytosine arabinoside (Ara-C), chlorpromazine, and mefloquine [110]. Cidofovir, a nucleoside analog, inhibits viral replication for its inhibitory action on DNA polymerase, but its effect has been tested with contradictory results [111,112]. Chlorpromazine inhibits clathrin-dependent endocytosis, which is required for JCV infection [113]. This drug is effective in limiting JCV infection in human brain-derived cell cultures, but has serious side effects and toxicity issues.
The antimalaric drug mefloquine was shown to inhibit JCV replication using *in vitro* cell culture models derived from human fetal brain in an attempt to identify drugs and biologically active molecules with antiviral activities against JCV [114]. Mefloquine has favorable pharmacokinetic properties and is known to cross the BBB and accumulate in the brain, where JCV infection is pathological, but has been associated with neurotoxicity [115]. Several independent case reports

showed that mefloquine treatment of PML was successful in reducing the viral burden in the brain and was associated with improvement of clinical symptoms [116]. However, as reported at the 2011 annual meeting of the American Academy of Neurology, a multicenter clinical trial supported by Biogen-IDEC Inc. and Elan Pharmaceuticals failed to show a reproducible reduction in the JCV DNA in PML patient CSF or reduced clinical progression of PML in response to mefloquine treatment [117].

However, the best treatment for PML is the restoration of the immune system, although even this is not ideal, since it can lead to IRIS. It is hence recommended that interventions made to restore the immune function, such as initiation of antiretroviral agents in HIV-infected individuals and removal of the offending chemotherapeutic agent, are made concomitantly to patients monitoring for the development of IRIS and treated with steroids accordingly [118].

Withdrawal from immunosuppressants in non-AIDS PML and HAART therapy in AIDS-related PML have been associated with immune reconstitution in the brain and control of viral replication. Rapid immune reconstitution is important to CNS immunosurveillance and control of JCV replication [reviewed in 119]; however, IRIS itself can be a serious, often fatal outcome.

Low T-cell counts and high numbers of copies of JCV DNA in the CSF at the time of PML diagnosis are clear risk factors for death [reviewed in 119]. Early use of five drug combined antiretroviral therapy after PML prognosis has been shown to improve survival, which is associated with recovery of anti-JCV T-cell responses and reduction of JCV DNA in the CSF [120].

Future therapeutics for PML should focus not only on blocking viral replication but also on reconstituting an effective T-cell response against the virus in the brain.

Treatment of PML occurring with newer biological agents: the fundamental step in treating confirmed or suspected PML in the setting of an offending agent is discontinuing the treatment and reversing the effect of the drug. In the case of Natalizumab treatment, 80% of binding sites remain occupied one month after administration and activity is still evident 3 months after discontinuation following two intravenous infusions separated by 4 weeks. Moreover, six months after cessation, an altered CD4/CD8 T-cell ratio in the CSF remains evident [reviewed in 101].

So, it is definitively important to eliminate the antibody from the body as rapidly as possible; plasma exchange allows decreasing the concentration of Natalizumab in the blood and desaturated α4 integrin.

1.1.2.5 Prognosis

Although PML is still a fatal disease and there is currently no specific treatment, the advent of HAART has altered the clinical picture of the disease for HIV-positive PML patients.

HIV-positive PML patients are living longer in the HAART era. Several studies have defined factors associated with PML prognosis in HIV positive patients. Clinically, a high CD4+ T-cell count, a low plasma HIV-1 RNA load, the presence of JCV cytotoxic T-cells, contrast enhancement on radiographic imaging and neurological recovery, and a low JCV load in CSF seem to be associated with longer term survival and with a good prognosis for PML patients [88,91,121,122]. In particular,

the amount of JCV viral load in CSF was inversely correlated to survival in a pilot study [123]. This was confirmed in a second study where a threshold of 50 to 100 JCV genome copies/µl of CSF was significantly associated with mortality [124].
Prior to the advent of HAART less than 10% of the HIV-positive patients was alive one year after the time of PML diagnosis [93].
To date, clinical stabilization of PML is seen in 50% of patients, and a survival rate as high as 63% has been reported, although survivors are often left with severe neurological sequellae [125].

1.1.2.5 PML and IRIS

Under treatment with HAART in HIV-positive patients with PML, the PML IRIS is commonly observed [126]. This disease condition is an inflammatory response to clinically apparent or subclinical pathogens, associated with recovery of the immune system after a period of immunosuppression, which leads to infiltration of lymphocytes into the PML lesions. An elevation of the CD8+ and CD4+ T-cell counts and the clinical appearance under treatment with HAART are used to diagnose PML-IRIS.
In PML, IRIS happens in two settings. The first is when symptomatic disease is treated with HAART and inflammation develops in relation to the existing lesions (paradoxical IRIS) [127]. In this setting, distinguishing a favourable immune reconstitution associated with clinical stabilisation from harmful IRIS associated with clinical worsening is important [128].
The second setting is when patients develop PML after the start of HAART and an inflammatory picture is found on MRI (unmasking IRIS) [126].
In PML-IRIS patients the clinical picture often aggravates due to the emergence of increased inflammation in the CNS. Inflammation can also lead to mass effects with herniation. In such severe cases the application of steroids can be beneficial. PML-IRIS can lead to a prolonged survival and even a cure from PML, when adequately managed [129].

1.1.2.6 PML and AIDS

PML is the cause of death in 3 to 5% of AIDS patients [85]. The incidence of PML complicating HIV/AIDS is higher than that of any other immunosuppressive disorder relative to their frequencies.
This may be due to several factors. First, the duration and extent of immunosuppression in HIV/AIDS may be greater compared with other immunosuppressive disorders [130]. Then, JCV specific CD4+ T-cell responses are impaired in HIV-infected patients with active PML compared to PML survivors on effective and prolonged antiretroviral therapy [131]. In addition, changes in cytokine secretion induced by HIV, viral interactions in coinfected cells, and increased BBB permeability facilitate the entry of B-lymphocytes infected with JCV into the brain [132]. Finally, there exist specific molecular mechanisms whereby HIV-1 may promote JCV gene expression, and participate in the pathogenesis of JCV [133].
The HIV-1 encoded regulatory protein Tat has been shown to increase transcription from JCV in glial cells by binding and enhance the JCV promoter [reviewed in 119], while the JCV agnoprotein may cause a slight decrease in the replication of HIV [134]. Tat protein mediates the activation of JCV early promoter

via a Tat-responsive transcriptional control element, named upTAR. Binding of Tar to upTar is mediated by the association of Tat with the cellular nucleic acid binding protein, Purα. Tat and Purα synergistically activate JCV late gene expression resulting in >100-fold induction [reviewed in 135].
Tat has also been shown to be secreted from HIV-1 infected cells and internalized by uninfected neighbouring cells, affecting cellular function [136].
Additionally, HIV infection increases permeability of the BBB [137]. The increased permeability may contribute to JCV crossing of the BBB in infected B-cells or as free virus. HIV proteins, such as Tat and Nef, can cause damage to astrocytes [138], and direct infection of astrocytes by HIV may lead to neuronal damage [139]. This damage may lead to increased inflammation and further infiltration by JCV infected lymphocytes and may help facilitate onset of PML.

1.1.2.7 PML and immunomodulatory treatments

Treatment with mAbs is a unique, newly identified predisposing factor for the development of PML. These therapies are promising for the treatment of a number of autoimmune conditions and lymphoproliferative disorders. Among the mAbs that increase the risk of PML are natalizumab (Tysabri), a α4β1 and α4β7 antagonist, and efalizumab (Raptiva), an anti-CD11a antibody.
Efalizumab is a humanized mAb against CD11a, a subunit of the leukocyte function-associated antigen type 1 (LFA-1), a T lymphocyte adhesion molecule, used in moderate to severe plaque psoriasis [140]. LFA-1 binds intercellular adhesion molecule 1 (ICAM-1), which allow migration of T lymphocytes from circulation into sites of inflammation. By binding the CD11a, efalizumab determined an alteration in apoptosis, cytotoxicity, cell proliferation, cytokine production, antigen presentation and gene activation [141]. This set of events affects psoriasis pathogenesis at multiple levels, in particular by inhibiting the initial T-cell activation in lymph nodes, preventing binding of T-cells to endothelial cells and blocking trafficking of T-cells from the circulation into the psoriatic skin preventing their reactivation in the dermal and epidermal layer [141].
Efalizumab was voluntarily withdrawal from the market in the spring of 2009 because of the occurrence of PML at incidence of approximately 1:500.
Rituximab has demonstrating efficacy in treating lymphoproliferative and a wide variety of autoimmune diseases, especially rheumatoid arthritis, SLE and MS [81,142,143].
In addition, PML cases have been observed after treatment with the small molecules mycophenolate mofetil, cyclophosphamide, fludaribine and azathioprine [85].
Mycophenolate mofetil is a selective, non-competitive and reversible inhibitor of inosine-5'-monophosphate (IMP) dehydrogenase, approved to prevent rejection of renal, cardiac and liver transplant. It has also demonstrated efficacy in the treatment of some autoimmune diseases, including SLE, autoimmune glomerular disease and myasthenia gravis. The molecule is metabolized by the liver to become mycophenolic acid, which blocks B and T-cell proliferation by inhibiting IMP dehydrogenase and preventing purine synthesis. The mechanism by which mycophenolate mofetil increase the risk of PML is unclear, but probably it is related

to its activity of T-cells depletion and lowering the immunological barrier for the disease by reducing or eliminating JCV cytotoxic T lymphocytes.
Ultimately, it can be stated that the occurrence of PML in relationship with compounds that limit the access of T-lymphocytes to the CNS underlines the importance of the celluklar component of the immune system in the control of JCV replication within the brain. Moreover, the occurrence of the pathology in the context of B-lymphocytes depletion suggests that B-cells are not the principal vehicle for JCV to enter the brain, and that humoral immunity might play a role in the control of JCV replication. On the other hand, B-cell depletion may impact on T-cell activity, which is known to be important in the control of JCV replication, and may stimulate the proliferation and the redistribution of the infected pre-B lymphocytes from the bone marrow to the bloodstream, favouring the viral entry into the CNS [144].

1.1.3 Not Determined LeukoEncephalopathy

After the introduction of HAART in the treatment of HIV-infected patients, the frequency of PML was not reduced as opposed to other opportunistic infections and, in addition, an increase in the occurrence of other form of HIV-related leukoencephalopathy has been reported. A PML-like leukoencephalopathy, indicated as Not Determined LeukoEncephalopathy (NDLE) is characterized by intense perivascular macrophage infiltration, extensive demyelination, and evidence of very high levels of HIV replication in the brain, in the absence of the JCV genome in the CSF.
In particular, neuropathologic changes characteristically associated with NDLE include formation of multinucleated gianT-cells, microglial nodules and astrogliosis in the white matter, basal ganglia and neocortex [145].
First Langford and colleagues [146] described autoptic findings of a severe form of NDLE, in seven patients who failed HAART. In the brain of thiese patients, they found only HIV, but no other pathogens. Moreover, the tissue injury in these cases was confined to the white matter, and the leukoencephalopathy they described was more severe than that described prior to the use of HAART.
They identified three stage of severity of this form of leukoencephalopathy, which were correlated with ante-mortem clinical, neuropsychological, and neuroimaging findings. These stages suggest a temporal sequence of pathologic events. In their model, leukoencephalopathy begins with perivascular infiltration by HIV-infected monocytes and BBB damage, leading to local HIV replication, moderate myelin loss, but limited gliosis. As disease progresses, more myelin is lost and is eventually accompanied by axonal damage and extensive astrogliosis.
While the etiology is unclear, the condition's occurrence in the post-HAART era in highly treatment-experienced individuals strongly argues that antiretroviral therapy plays a basic role in pathogenesis.
HIV-associated leukoencephalopathy may simply be a consequence of antiretroviral therapy that extends the survival of patients with HIV-encephalopathy without completely controlling HIV replication in the brain. Alternatively, its etiology may be more complex, involving other HIV-, immune-, and drug-mediated effects.

1.2 The Human Polyomaviruses

The family name *Polyomaviridae* derives from the observation that the first member of this family could induce multiple (poly) tumors (oma) in mice [147]. Polyomaviruses were historically categorized with the Papillomaviruses under the designation of Papovaviruses until the separation into two distinct families in 2000 [148].

Polyomaviruses (PyVs) are small, naked viruses with icosahedral capsid and circular, double-stranded DNA genomes and they have been isolated from a number of species, including human, monkey, rabbit, rodents, and birds. Their host range is rather restricted, and they generally do not infect other species productively [149]. Simian virus 40 (SV40) and mouse Polyomavirus are the prototypical and most well characterized member of the *Polyomaviridae* family [150].

Human are the natural host for two members of the *Polyomaviridae* family, JCV and BK virus (BKV), both isolated in 1971 from the urine of a kidney transplant recipient with the initials B.K, who developed uretheric stenosis, and from the brain tissue of a Hodgkin lymphoma patient with the initials J.C. who suffered from PML, respectively [151,152].

JCV is defined as the etiological agent of PML. BKV has been identified as the main cause of Polyomavirus-associated nephropathy (PVAN), the major cause of renal allograft failure.

Recently, eight novel human Polyomaviruses (HPyV) have been identified. In 2007, the HPyV KI and WU were characterized in nasopharyngeal aspirates of children with acute respiratory symptoms at the Karolinska Institute and at the Washington University, respectively [153,154].

In 2008, Merkel cell Polyomavirus (MCPyV) was isolated from a rare skin tumor named Merkel cell Carcinoma (MCC) [155], and in 2010 the novel HPyV6 and HPyV7 were characterized from skin swabs of healthy individuals [156], though they have not been associated with any human disease. Trichodisplasia spinulosa-associated Polyomavirus was identified in a boy suffering from this rare skin disease characterized by the development of follicular papules and keratin spines [157]. Viral DNA sequences of HPyV9 were amplified from a serum of a kidney transplant patient [158], and, lastly, HPyV10 was isolated few month ago from condyloma specimens of a patient with warts, hypogammaglobulinemia, infections, and myelokathexis syndrome [159].

Primary infection with both BKV and JCV is usually asymptomatic. BKV commonly infects young children and adult seroprevalence rates of 65-90% being reached by the age of ten years. In contrast, the pattern of JCV infection appears to vary between populations; in some anti-JCV antibody is acquired early as for BKV, but in others anti-JCV antibody prevalence continues to rise throughout life. This indicates that the two viruses are probably transmitted independently and by different routes. While BKV DNA is found infrequently in the urine of healthy adults, JCV viruria occurs universally, increasing with age, with adult prevalence rates often between 20% and 60% [160].

The primary infection is followed by a lifelong, subclinical persistence of episomal viral genomes in targeT-cells, and in the context of profound immunosuppression

the virus infection can be activated, leading to the destruction of tissues and diseases [161].
BKV and JCV can be detected in a variety of tissue, but BKV is considered a nephrotropic virus, whose-associated illnesses occur primarily in the urinary tract, and JCV a neurotropic virus, since its pathogenic effects are confined exclusively to the CNS.
Both viruses have oncogenic potentials in animal models and cell culture, and viral DNA/ARNA and proteins can be detected in human tumors. However, their role in human cancer remains controversial [reviewed in 162].

1.2.1 Viral structure and genome

JCV and BKV display a high degree of nucleotide sequence homology, of about 75%, but, in spite of their high resemblance, these closely related polyomaviruses exhibit distinct biological behavior and disease pathogenesis [163,164].
Polyomaviruses are composed of small, non-enveloped, ichosaedral virion, measuring 40-45 nm in diameter and comprising 88% proteins and 12% DNA. The capsid is composed of three virus-encoded structural proteins, Viral Protein 1, 2, and 3 (VP1, VP2 and VP3). VP1, the most highly homologous among JCV and BKV, is the major component with 360 molecules per capsid, and VP2 and VP3 contribute with 30-60 molecules each to the capsid. The ichosaedron consists of 72 pentamers with no apparent hexamers, each composed of five VP1 molecules and one molecule of VP2 or VP3. Only VP1 is exposed on the surface of the capsid, and this determines the receptor specificity [119,149].
The capsid surrounds a single, super-coiled, double-stranded DNA molecule of 5130 base pairs (bp) in the case of the prototype JCV genome Mad-1 strain or 5153 bp as seen in the BKV Dunlop strain. The viral genome is associated with cellular histones H2A, H2B, H3 and H4 to form the so-called minichromosome, structurally indistinguishable from host cell chromatin; the viral particles do not contain linker histones, but the genome acquires them after entry into the host cell [119,150,149].
The viral genome of JCV and BKV is functionally divided into three regions, called the genetically conserved early and late coding regions, of about the same size, which are separated by the hypervariable non-coding control region (NCCR), containing the origin of viral DNA replication (ori), the TATA box, binding sites for cellular transcription factors and bidirectional promoter and enhancer for the transcription of early and late genes. The NCCRs of polyomaviruses are the most variable portions of the viral genome within a single virus as well as across genera of viruses
Viral DNA transcription and replication occurs bidirectionally starting from the NCCR: the early transcription proceeds in a counterclockwise direction, while the late transcription proceeds clockwise on the opposite strand of DNA [135].
The early coding region spans about 2.4 kb and encodes the alternatively spliced transforming proteins large tumor antigen (T-Ag) and small tumor antigen (t-Ag), which are involved in viral replication, and in promoting transformation of cells in culture and oncogenesis in vivo. T-Ag, a nuclear phosphoprotein of approximately 700 amino acids (aa), is considered the master regulator of the infectious process, because it orchestrate the production of early pre-messenger RNA (mRNA), the

initiation of viral DNA replication and the activation of late gene transcription. Moreover, binding to the hypophosphorylated form of the retinoblastoma susceptibility protein (pRb), T-Ag allows for premature release of the transcription factor E2F, which stimulate resting cells to enter the S-phase of the cell cycle. Thus, T-Ag directly recruits the host cell DNA polymerase complex to the ori in order to initiate bi-directional DNA synthesis. Activation of the late viral promoter by T-Ag and associated cellular transcription factors leads to viral late gene expression [reviewed in 150].
t-Ag is a cysteine-rich protein of 172 aa in BKV and JCV, the first 80 of which are shared with T-Ag. t-Ag role in the lifecycle of human polyomaviruses is not yet fully understood, though it is believed to serve an ancillary role fot T-Ag activity and cell transformation [135,165].
Additional T antigen isoforms have also been identified in JCV and BKV: JCV encodes three T' antigens, T'165, T'136, and T'135 [166] and BKV encodes a truncated T antigen close in structure to T'136 of JCV [167]. The late coding region spans 2.3 kb and contains the genetic information for the major structural protein VP1 and the two minor structural proteins VP2 and VP3, that are encoded from a common precursor mRNA by alternative splicing. The late region also encodes the agnoprotein, which role in the polyomavirus life cycle remains under investigation, but appears to be multifunctional, and varied functions have been attributed to it, ranging from viral transcriptional regulation to inhibition of host DNA repair to functioning as a viroporin [150,168].

1.2.2 The infectious lifecycle of polyomaviruses

Receptors: the infectious lifecycle of polyomaviruses is divided into an early and a late stage. The early stage begins with the initial interaction of the virus with the surface of the host cell and continues until the onset of viral DNA replication. The late stage includes all events that lead to the assembly of new virions and concludes with the release of viral progeny, which represents the completion of the entire viral replication cycle [150].
The infection of cell by polyomaviruses is initiated by the interaction of VP1 with specific receptors and co-receptors. It is known that BKV uses gangliosides GD1b and GT1b for the initial interaction with susceptible cells. More recent experiments argue that an N-linked glycoprotein with α(2,3)-linked sialic acid serves as the receptor for BKV on host cells. The identity of the proteinaceous component of the cellular receptor(s) for BKV remains to be determined [reviewed in 150].
JCV also requires sialic acid to infecT-cells and has been reported that it uses both α(2,3)- and α(2,6)-linked sialic acids to infect permissive glial cells [169]. Recently, a receptor moiety used by JCV for productive infection has been identified and a recombinant JCV VP1 pentamer has been shown to bind specifically to lactoseries tetrasaccharide C (LSTc), which contains a terminal α(2,6)-linked sialic acid. It appears likely that JCV primarily uses α(2,6)-linked sialic acids to bind to cells, with the possibility that the weak interactions between JCV and α(2,3)-linked sialic acids play a limited role in infection. It is currently unknown onto what receptor molecule the LSTc moiety is attached [170].
In addition to using sialic acid as a receptor, JCV has been shown to require the serotonin receptor, $5HT_{2A}$, to infect glial cells [171].

The $5HT_{2A}$ receptor protein contains several potential glycosylation sites, but it is unclear whether LSTc or other sialic acid moieties responsible for infection reside on this protein. Elimination of glycosylation sites on $5HT_{2A}$ receptor obstructs receptor expression of the cell surface, preventing a clear consensus on the need for sialic acids on $5HT_{2A}$ for infection. Additionally, gangliosides may play a role in JCV infection, and the ganglioside GT1b has been reported to function as a receptor for JCV [119,172].

Mechanisms of entry and intracellular trafficking: BKV and JCV penetrate into the cytoplasm by endocytosis, employing different internalization mechanisms to enter the host cell.

BKV enters cells by caveolae-mediated endocytosis with a relatively slow process, while JCV enters rapidly and requires proper assembly of clathrin-coated pits. Inside the cell, BKV co-localized with the caveolae marker Cholera toxin subunit B and JCV with the clathrin marker, transferrin, in endosomes.

After penetration into the interion of the cell, BKV and JCV are next transported through the cytosol to the nucleus, depending on the active cytoscheletal transport machinery. Close examination of the involvement of the cytoscheletal components demonstrated a critical role for intact microtubules during early infection by both BKV and JCV.

In the nucleus: uncoating of polyomavirus occurs inside the nucleus, the site of viral replication and virion assembly. Here, the viral lifecycle is regulated by a temporal setting, according to which there is primarily the transcription of the early viral genes T-Ag and t-Ag upon entering the nucleus, followed by a switch to DNA replication. Afterwards, there is a second switch to the expression of late viral genes, VP1, VP2, VP3 and agnoprotein.

In the nucleus of a permissive cell, the primary transcript is expressed from the early region and is alternatively spliced to give two mRNAs encoding T-Ag and t-Ag. Subsequent to early transcription is the switch to DNA replication, primarily initiated by T-Ag, which complexes with host cell DNA polymerase α-primase and replication protein-A at the viral ori, and unwinds the double helix, promoting DNA synthesis [173].

Polyomaviruses rely on cellular enzymes and cofactors for DNA replication. Since these proteins are expressed in the S-phase of the cell cycle, T-Ag modulates cellular signaling pathway and stimulates the cell cycle, though its ability to bind a number of key cellular proteins that control cell cycle progression and apoptosis. In this manner T-Ag can direcT-cells to enter the S-phase thus enabling DNA viral replication.

Once the viral genome has been replicated, T-Ag mediates repression of early gene expression, and stimulates transcription of the late genes. Finally, the capsid proteins VP1, VP2, VP3, expressed from the late region and localized into the nucleus, assemble with the replicated viral DNA to form a newly packaged virion progeny, which are released upon cell lysis [174] (fig. 8).

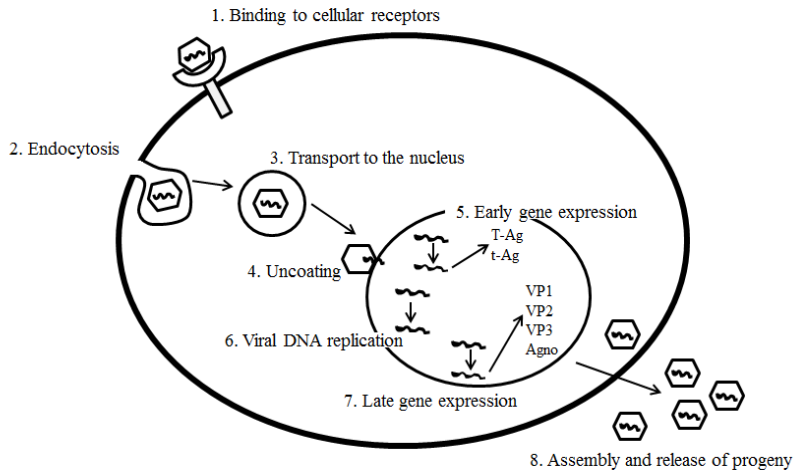


Fig. 8: life cycle of polyomaviruses in a permissive cell. The following steps are indicated: 1: binding of the viruses to cell surface receptors; 2: internalization into the cytosol by endocytosis; 3: transport to the nucleus; 4: uncoating; 5: transcription of the early region; 6: viral DNA replication; 7: transcription of the late region; 8: assembly of the viral progeny in the nucleus and new virions releasing from the cell.

1.2.3 JCV

JCV is a ubiquitous human polyomavirus that cause primary infection during late childhood, typically subclinical or linked to a mild respiratory illness: in some urban areas the rate of JCV seroconversion may exceed 90%. Between the age of 1 and 5 years, approximately 10% of children demonstrate antibody to JCV, and by age of 10 years it can be observed in 40-60% of the population. By early adulthood, as many as 70-80% of the population has been infected.

The mode of transmission for JCV is not yet well defined, however, the presence of JCV DNA in B-cells and stromal cells of the tonsils and oropharynx supports the hypothesis of a respiratory transmission, with tonsils serving as the potential site for initial infection [175]. JCV is found also in raw sewage, suggesting that ingestion of contaminated water or food could be another portal of virus entry [176-178], and in a high percentage of normal tissue samples taken from the upper and lower human gastrointestinal tract [179,180].

Once the primary childhood viremia is restricted by the action of immune system, particularly cell-mediated immunity, the virus is proposed to remain in the body in a state referred as viral latency, with periodic re-expression during periods of immunosuppression. The JCV latent state is not well understood but is thought to involve an asymptomatic, chronic, persistent infection wherein JCV DNA but no proteins are detected. Presumably, the circular viral genome is present as an

episome (latency), or alternatively viral DNA replication may occur sporadically or intermittently at a level below the threshold of detection (persistence), and this may depend on the tissue [84].
Many tissues have been reported to harbor latent JCV, including kidney, though to be the major organ of JCV persistence during latency, tonsils, spleen, lymph node, lung, bone marrow, and certain type of peripheral blood leukocytes, including B lymphocytes. Importantly, it has been reported the presence of JCV in the brain of healthy individuals without PML. Regarding the kidney, active viral replication is probably occurring episodically in the urothelium, because virus can sometimes be detected in the urine [84].
JCV, as the other human polyomavirusess, causes significant disease primarily in individuals that are immunocompromised, allowing reactivation from the persistent subclinical state to a lytic infection resulting in viruria and viremia, potentially leading to severe or fatal diseases [181]. The most common underlying cause of immunosuppression leading to JCV reactivation is AIDS. Reactivation results in the lytic infection of oligodendrocytes in the brain and the development of the fatal demyelinating lesions observed in the case of PML.
Other immune-altering conditions, in which cases of PML have been reported, include lymphoproliferative diseases, such as lymphomas and leukemias, myeloproliferative diseases, organ transplants, chemotherapy, inherited immunodeficiencies, and treatment with immunomodulatory compounds, mainly some monoclonal antibodies [130,182,183].
There has been much debate on the nature of latency and its site(s) within the body, and it is still incompletely understood how the virus infects the CNS. In summary, there are two main hypotheses [84]. First, JCV latency and reactivation involves immune cells, especially B-lymphocytes in bone marrow, which acts as a source of latent virus and allow virus to circulate around the body and enter the brain [85,90]. Second, JCV DNA may be present in the brain prior to PML, and event(s) related to immunosuppression or HIV-1/ AIDS initiate viral replication; that is, the brain is the site of JCV latency and reactivation. In the CNS, JCV is possibly controlled by T-cells that migrate there. In the situation of an alteration of the immune system, possibly this control of the virus in the CNS is reduce and viral infection emerges. In addition, infection with different viral variants during a lifetime is not excluded and some of these variants are more likely to lead to CNS infection in the situation of a compromised immune response. Although no clear consensus has emerged about the nature and site of JCV latency, these two hypotheses are not mutually exclusive [90,129].

1.2.3.1 JCV genome

Similar to the case for all other polyomaviruses, the JCV genome is a closed, circular, supercoiled DNA molecule that is composed of early and late genes, separated by the 400 pb NCCR or untranslated transcriptional control region (TCR), which contains the origin of replication, promoter, and enhancer elements (fig. 9) [184]. The early region is expressed *de novo* after infection and before DNA replication and is on the ori-proximal side of the NCCR. Late genes are optimally expressed concurrently with or after DNA replication and are found on the ori-distal side of the NCCR [reviewed in 119].

The NCCR lies between the early and late coding sequences and is thought to be the main determinant of cell type specificity. Indeed, sequence modification in this promoter/enhancer structure can alter the cellular host range and may allow JCV to switch between states of lytic and latent infection.
NCCR is composed of fairly well-conserved flanking regions that border the transcription start sites of the early and late coding regions, as well as a central region containing numerous transcription factor binding sites [119,185].
The NCCR varies greatly between isolates from PML patients. In addition, a sequence, known as archetype, has been isolated from urine specimens from both PML patients and healthy people but is rarely found in PML brain tissue. The original sequence isolated from the brain of a PML patient is known as Mad-1, as it was isolated at the University of Wisconsin- Madison.

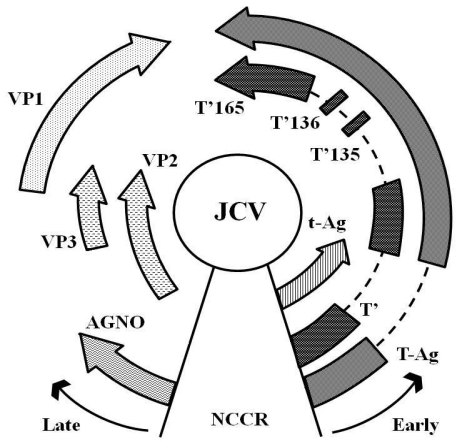


Fig. 9: schematic representation of JCV genome organization. The circular, double-stranded DNA genome is ~5.2 kb in size and is divided into the early coding region and the late coding region, transcribed in opposite directions from a common NCCR. Early genes include T-Ag, t-Ag, T'135, T'136 and T'165. Late genes include agnoprotein, VP1, VP2 and VP3.

Naturally occurring variants: the JCV regulatory region can be divided into 7 distinct sequence sections, all containing promoter/enhancer elements. The 128 bp region that immediately precedes the early coding sequences includes the origin of viral DNA replication. Opposite the direction of early transcription, starting 12 bp downstream from the center of the ori, sequence sections designated as *a* (25 bp), *b* (23 bp), *c* (55 bp), *d* (66 bp), *e* (18 bp), and *f* (69 bp) constitute the so called archetype sequence (fig. 10). The archetypal form of the virus is found in the kidney and urine of healthy and immunosuppressed individuals. It is typified by the CY strain of JCV and may represent the transmissible form of the virus. Despite the

presence of functional protein-coding regions and an ori, archetypal NCCRs do not support robust growth in culture [90,185].
Repeats and/or deletions from the archetype sequence account for all other known regulatory region variants [186].

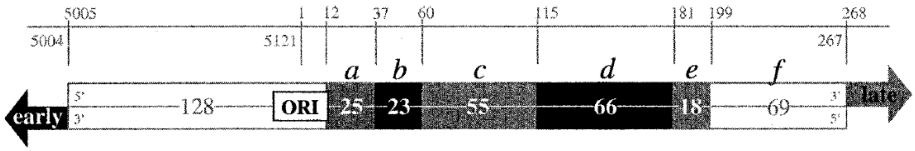


Fig. 10: linear representation of JCV archetype NCCR sequence from [185]. Modified nucleotide-numbering system adapted from the prototype JCV genome, Mad-1 (Frisque et al, 1984). The archetype NCCR contains a single copy of all sequence sections observed in all other variant forms of JC virus regulatory region. From the early side, the initial regulatory region sequence section contains the ori followed by sequence sections designated a, b, c, d, e, and f . The base-pair length of each sequence section is noted.

When present, the sections *b* and *d* have been shown to inhibit the efficient transcription and replication of JCV. Duplication of any singular grouping of section *a* through e in tandem sequences is thought to enhance level of viral activity, even if the tandem sequences experience substantial nucleotide deletions. The *f* sequence section usually follows any singular or repeated grouping of sections *a* through *e* and precedes the late coding regions [185].
The regulatory region amplified from PML brain and CSF usually show rearrangements generated by point mutation, base sections insertions and deletions compared to the archetype. In particular, the NCCR from the original isolate of JCV, Mad-1, is characterized by two deletions and a duplication, in comparison to the archetype. It contains an enhancer element, which exist as a 98 bp tandem repeat, and duplicated TATA boxes as well as multiple transcription factor binding sites (fig. 11). The tandem repeat structure of the Mad-1 NCCR variant has been termed the prototype JCV NCCR sequence and is composed of the three sequence sections *a*, *c* and *e*, with the TATA boxes found in *a*. The Mad-4 variant is identical to Mad-1 except that the NCCR contains a 19-bp deletion that eliminates the second, late-proximal TATA box.
In addition, numerous NCCR variants containing a tandem repeat-like structure, and generally termed rearranged forms, have been isolated from tissues of patients with PML. These forms present some level of sequence section *b* and *d* integration, but also contain repeated sequences. Although Mad-1 was the first sequenced NCCR isolated from a PML patient and is referred to as the prototype, it appears to be a somewhat atypical isolate. The tandem repeat sequences serve as

a good reference point and newly isolated JCV sequences are generally compared to the Mad-1 variant.

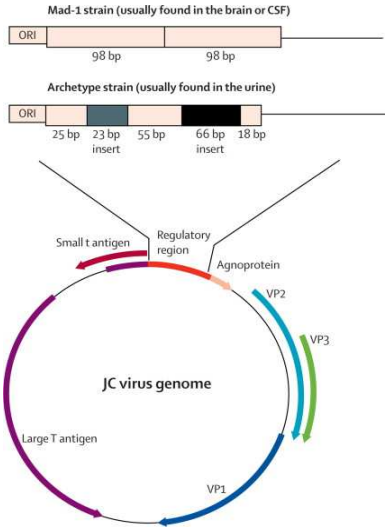


Fig. 11: the JCV NCCR detected in the brain or CSF of patients with PML usually consists of tandem repeats of a 98 bp element, as in the Mad-1 strain; most isolates from the urine of healthy individuals and patients with PML are similar to the archetype regulatory region (quiescent in the kidneys after asymptomatic primary infection). The JCV archetype has one 98-bp element with a 23-bp insert and a 66-bp insert.

Because the prototype NCCR contains a repeat structure, it contains significantly more binding sites for transcription factors essential to viral gene expression. In particular, the archetype sequence does not contain the Oct-6/tst-1/SCIP sites present on the border between regions *c* and *e*. Additionally, the lack of neighboring *a* and *c* regions eliminates Spi-B binding sites, which are important for early viral gene expression [187]. The lack of binding sites for brain-specific transcription factors may be what abrogates the ability of archetype virus to cause disease in the brain. The lack of repeats of region *c* in archetype also leads to a reduced number of NFI binding sites, which allow a family of transcription factors to bind the JCV genome and are essential for fully activating viral transcription in the brain and cells of the lymphoid system. These results indicate the importance of selective repeated binding sites for the cellular transcription factors involved in activating viral gene expression.

The mechanism for derivation of prototype sequence from archetype and dissemination of archetype in the host have yet to be demonstrated, although the prevailing model holds that archetype-like sequences are transmitted from person

to person and then undergo deletions and duplications within the infected host, leading to PML-type NCCR sequences, which traffic to the brain. This rearrangement of the NCCR may take place in lymphoid cells like B-cells, since they possess the Rag1 and Rag2 enzymes for immunoglobulin gene rearrangements.
In order to classify newly sequenced viral NCCRs, a compass-like classification scheme that organizes NCCR sequences into four distinct variant types was developed [185]. Variant type I NCCRs contain no inserts in the *a-c-e* organization of the NCCR and can be divided into variant type I-S (singular *a-c-e*) and variant type I-R (*a-c-e* repeat with no inserts) (Mad-1 and Mad-4). Variant type II NCCRs contain inserts into the *a-c-e* sequence and can be classified as variant type II-S (*a-b-c-d-e*, or archetype-like) or variant type II-R (containing inserts and repeats) (e.g., Mad-7 and Mad-8) (fig. 12 and table 1).

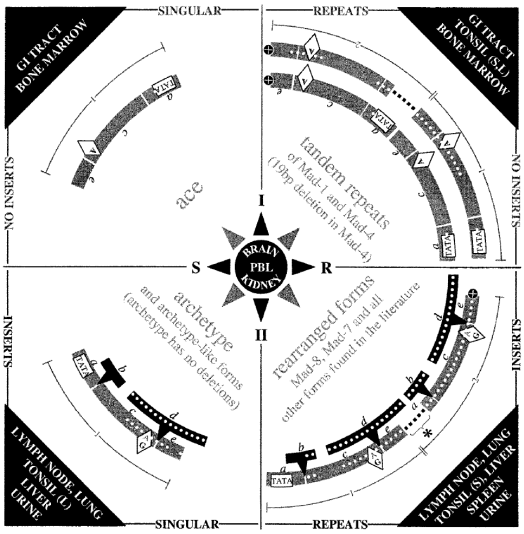


Fig. 12: the Compass: a schematic diagram of the relationships between JCV regulatory region sequences published worldwide (adapted from [185]). JCV variant NCCR grouped into quadrants (I-S, I-R, II-S, and II-R). Upper quadrant variant types (I) have no additional sequence integrated into the a-c-e units (no inserts). Lower quadrant variant types (II) have dark integrated sequence sections (inserts), b (23 bp) and d (66 bp). Both types I and II are divided into singular (S) and repeat (R) forms by the left and right quadrants, respectively. Dots represent sites of possible bp deletions.
Right quadrants (R-forms) have dark dashes where sequence is deleted and + where additional repeats may occur. JCV tropism common to all variant regulatory region forms is contained in dark central circle. Specific JCV tropisms are contained in dark corner triangles.

Table 1: NCCR variants.

Variant Type	*NCCR Structure*	*Tissue Association*	*Example*	*NCCR sequence block variations**	*Disease association*
IS	Singular, no insert	GI tract, bone marrow		*a c e* *f*	Colon cancer?
IR	Repeats, no insert	GI tract, tonsil, bone marrow	Mad-1	*a c e* *ace* *f*	PML
IIS	Singular, inserts	Lymph node, lung, tonsil, liver, urine	Archetype	*a*bcd*e* *f*	Urine (non-pathogenic)
IIR	Repeat, inserts	Lymph node, lung, tonsil, liver, spleen, urine	Mad-8	*a*bc *+e* *bc+e* *f*	PML

* +, one base insertion; lowercase letter, full sequence block; italic *b*, partial sequence block. All variants are found in the brain and peripheral blood lymphocytes.

Viral genotypes: VP1 genotyping region is the other polymorphic part of the JCV genome, first recognized by serology and restriction fragment length polymorphisms and then defined by sequencing of different isolates and strains by which at least eight different viral genotypes and several subtypes have now been described.

Since JCV transmission from parent to offspring allow the same genotype to persist for generations within a population, many genotyping studies have been carried out in diverse population group to associate different types of JCV with populations of various descent as well as to map population movements. It has been hypothesized that type 6 is the original JCV type and that JCV coevolved with human populations.

Type 1 and type 4 are generally associated with Europeans and European-Americans, while type 2A is found generally in Asians and Native American populations. Types 3 and 6 are isolated primarily from Africans and African-Americans. Types 2D and 7C are found among both Asians and South Asians [188,189]. Types 2E, 8A, and 8B are found in Western Pacific populations [189].

Interestingly, type 8A is found only in populations of Papua New Guinea [190]. JCV subtype 2B, which is more often found among Asians and Eurasians, is reported to be the most frequently found in the brain of PML patients than expected on the basis of its prevalence in urine samples from a control normal population; on the contrary, type 4 has been associated with lower disease risk [191] (table 2).

More recently, investigation of changes in viral DNA coding regions from brain biopsy specimens and CSF specimens from PML patients has been undertaken. Alterations in structural protein amino acid sequences could lead to enhanced viral entry and thus contribute to PML or, alternatively, could potentially cause distinct diseases. Some evidences point to the association of mutations of certain amino acid residues in the region of the sialic acid binding sites and surface loops of VP1 with PML and that these mutations occur within the patient after initial infection with JCV [reviewed in 119].

Table 2: VP1 types and associated ethnic groups (from [119]).

Type	***VP1 type change(s) from consensus[a]***	***Predominantly associated ethnic group***	***Identical VP1 sequence***
1A	75R, 117S, 158L, 345K	European/ European American	
1B	74S, 117S, 126A	European/ European American	
2A1	113(L), 117(A), 126(A), 164(T)	Asian/ Native American	
2A2	115L	Asian/ Native American	
2B[b]	126A	Asian/ Eurasia	
2D1	Consensus	Asian/ South Asian	7C1, 7C2
2D2	126A	Asian/ South Asian	2B
2E	113L, 321I	Western Pacific populations	7A
3A	134A, 164T, 321I, 332Q	African/ African American	3B
3B	134A, 164T, 321I, 332Q	African/ African American	3A
4[c]	134A, 164T	European/ European American	
6	164T	African	
7A	113L, 321I	Asian	2E
7B1	113(L)	Asian	
7B2	37V, 321I	Asian	
7C1	Consensus	Asian/ South Asian	2D1, 7C2
7C2	Consensus	Asian/ South Asian	2D1, 7C1
8A	12H, 164T	Inhabitants of Papua New Guinea	8B
8B	12H, 164T	Western Pacific populations	8A

[a]Parentheses indicate that the change is found in less than 50% of the type; [b]slightly higher PML risk; [c]slightly lower PML risk.

1.2.3.2 JCV tropism

The strict species specificity of JCV for humans has confounded the development of animal models for PML.

In vivo, JCV infection is likely restricted to kidney epithelial cells, tonsillar stromal cells, bone marrow-derived cell lineages, oligodendrocytes, and astrocytes [reviewed in 119].

The virus is thought to establish low-level persistent or latent infections in the kidney and in bone marrow-derived cells largely due to inefficient viral replication in these cell types. Once in the central nervous system (CNS), the virus replicates vigorously in oligodendrocytes, leading to the demyelinating disease PML.

The cell type-specific tropism of JCV observed *in vivo* is mirrored *in vitro*, with virus productively infecting bone marrow-derived cells, tonsillar stromal cells, and macroglia [reviewed in 119]. Virus replication is maximal in primary human fetal glial cell cultures and in some human glial cell lines [193], as well as in other cell lines expressing SV40 T-Ag, such as COS-7 cells [192,193].

The major tropism of JCV for human glial cells is not fully understood, but multiple factors are likely responsible for contributing to robust viral replication in this cell type. Host cell- and species-specific transcription and replication factors contribute significantly to the restricted specificity displayed by JCV and other members of the genus [194,195]. In addition, virus receptor interactions contribute to viral tropism and spread.

Tropism for glial cell may be determined at the level of viral early gene transcription, which is responsible for the production of T-Ag protein. Several lines of evidence indicate that the JCV early promoter is preferentially expressed in glial cells. For example, recombinant DNA construct, in which the JCV early promoter is

linked to a reporter gene, show much higher level of expression in glial cells than in non-glial cells [196]. Transgenic mice with the JCV early region selectively express T-Ag in oligodendrocytes leading to a phenotype of dysmyelination [197]. In addition, transgenic mice with constructs in which the early promoter and T-Ag regions of JCV and SV40 were exchange showed patterns of expression that demonstrate the JCV early promoter, and not the T-Ag coding sequence, is responsible for glial cell expression [198]. In vitro transcription assay showed that hamster glial cell extract stimulated the JCV early promoter whereas HeLa cell (an immortal cell line derived from cervical cancer) extract reduced production of nuclear run-off transcripts. This suggests that glial cells contain one or more an activating factors for transcription of JCV early promoter, while non-glial cells contain a transcriptional repressor [199,200].

1.2.3.3 Oncogenicity of JCV

The first evidence on the association of JCV with cancer came from reports of brain tumors found in patients with concomitant PML.

JCV is able to transform cells in culture, particularly cells of glial origin including human fetal glial cells and primary hamster brain cells. JCV-transformed cells exhibit the phenotypic properties associated with transformation including growth in soft agar, serum-independence, changes in morphology, plasminogen activator production, etc. The transforming ability of JCV appears to be limited to cells of neural origin and cell-type-specific transcriptional regulation of the viral promoter is thought to be responsible for this property [201].

Many studies have established the highly oncogenic potential of JCV when injected into laboratory rodents and JCV is the only human virus that induces solid tumors in non-human primates. Transgenic mice with the JCV T-Ag gene develop a variety of neural tumors. In humans, there have been many reports on the presence of JCV DNA sequences and T-Ag expression associated with some brain and other tumors [135]. The presence of JCV is been investigated in a broad range of glial-origin tumors including oligoastrocytomas, astrocytomas, anaplastic astrocytic, anaplastic oligodendroglial, glioblastomas and ependymomas. JCV DNA has been detected in nearly every known type of malignant and non-malignant tumors which arise from cells of the CNS [202].

It is interesting to note that JCV DNA and viral T-Ag protein has been detected at very high percentages, at similar seen in the CNS-origin tumors, in the colon tumors. The prevalence of JCV sequences and T-Ag in these tumors suggests that these cell types are permissive for the expression of the JCV early promoter, which may lead to the accumulation of T-Ag and potential cellular transformation. This evidence, taken together with other potential sites of latency in humans, including B-cells and kidney epithelium, suggest areas of further study to investigate the presence of JCV in normal and tumor tissues of non-CNS origin [202]. T-Ag and t-Ag are the viral molecule that mediate the oncogenic potential of JCV. The T-Ag is thought to be the primary agent in transforming cells, and it interacts with a plethora of cellular proteins that are responsible for driving cells into S phase. Two of these regions alter the pRb related tumor suppressor proteins, which normally binds to transcription factor E2F. Free E2F activates transcription of genes involved in DNA synthesis, while binding of E2F by pRb represses these genes.

Phosphorylation of pRb releases E2F, and leads to progression of the cell cycle and growth. The J domain of T-Ag, located near the N-terminal, affects pRb by mediating degradation of pRb complexes. The N-terminus contains another domain which binds to these pRb complexes.
A region of T-Ag has the ability to bind p53, whose normal function is to detect DNA damage in the cell and either pause the cell cycle or to activate an apoptotic program. Aside from these pathways, there is evidence that T-Ag can alter the human chromosome in other unspecified ways.
The t-Ag has a homologous N-terminus when compared with T-Ag, but it has a unique C-terminal end. It has been suggested that t-Ag may help transform cells during times of stress, such as nutrient starvation, which would normally suppress proliferation.

1.2.3.4 JCV pathogenesis

JC virus infection is species-specific and it is only found in humans. Therefore, research on JCV pathogenesis has been hampered by the lack of an animal model. While all JCV infected oligodendrocytes appear to sustain a productive infection, some astrocytes do also harbor late JCV genes and are destroyed, while other may sustain an abortive infection and appear transformed [87]. A model for JCV pathogenesis is shown in fig. 13: the initial site of infection is assumed to be tonsillar stromal cells, from which JCV is disseminated by lymphocytes to bone marrow CD34+ cells and uroepithelial cells in the kidney, presumed sites of latency. Reactivation occurs during immunosuppression or immune modulation, after which it gains entry into the brain by way of peripheral B lymphocytes. Susceptible cells (oligodendrocytes, astrocytes, and progenitor cells) share a characteristic expression pattern of nuclear transcription factors required for synthesis of JCV early viral protein mRNA and replication of JCV DNA.

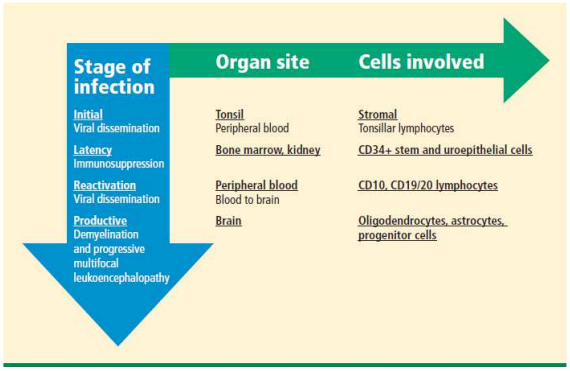


Fig. 13: a model for pathogenesis of JCV infections (from [203]).

1.2.3.4.1 JCV and PML

As described above, primary infection of JCV is asymptomatic and occurs in immunocompetent individuals early in childhood. Profound immunosuppression provides suitable condition for increase in JCV replication and spread to the CNS, where the virus selectively targets and destroys the myelin-producing glial cells, the oligodendrocytes. Immune compromising condition includes AIDS, lymphoid system cancers, such as lymphomas, or long-term immunosuppressive therapy for treatment of cancer or autoimmune diseases [119,152,204].

Patients who undergo organ transplant are also at risk due to the need for chemotherapy to prevent organ rejection [205]. Asymptomatic viruria can occur in old age, pregnancy, and diabetes mellitus, presumably as a result of hormonal effects on anti-viral immunity [206].

The loss of oligodendrocytes results is a patchy and subsequent confluent demyelination of the CNS referred to PML [83]. Development of PML proceeds as a stochastic event. Based on current incomplete understanding, it is possible to speculate a plausible scenario for PML development (table 3).

Following the primary infection, JCV disseminates and establishes latency in various sites within the body. Latency in the brain remains controversial because some investigators have detected JCV DNA but not protein expression [207], and others have found the T-Ag, but rarely [208]. Based on the pervasive presence in urine of the archetype JCV, it is likely, but not proven, that the primary infection occurs with this strain, incapable of an efficient replication in glial cells. It would then require genetic modification, in particular a 98 bp tandem repeat in its regulatory region to become neurotropic.

Table 3: the proposed stages of development of PML (adapted from [209]).

	Initial infection of JCV
1.	Establishment of JCV latency, most importantly in CD34+ and other B-cell lineages
2.	Release of B-cells (immature and pre-B-cells) from bone marrow: a) re-activation of JCV within this immature B-cells due to viral transactivation by transcriptional factors that are released during B-cell maturation b) Mutation of JCV to a neurotropic form within the B-cells that have the unique genetic machinery to permit this, namely, machanisms for gene rearrangement, the addition or deletion of nucleotides to the genome, and somatic hypermutation
3.	Actively replicating neurotropic JCV in the circulation
4.	Brain entry of neurotropic JCV
5.	Establishment of productive infection of oligodendrocytes
6.	Impairment of CNS immunosurveillance: a) Inability of JCV-specific tcitotoxic T-cells to enter the brain b) Inability to process JCV antigen locally in the brain due to the depletion of perivascular dendritic cells

It has been proposed that B lymphocytes, besides serving as a potential site of viral latency, may play an important role in the pathogenesis of PML. Since they can undergo a V(D)J recombination and have a unique genetic machinery that

facilitates gene rearrangements, it is possible that they could harbor recombination and/or rearrangements of viral genome (fig. 14).
This hypothesis is sustained by the observation that diverse viral NCCRs, including archetype-like and prototype-like NCCRs, have been found in the blood and bone marrow [reviewed in 119, 210,211].
During the latency into the B lymphocytes, it is possible that the virus is periodically re-expressed, ultimately trafficking to the brain and establishing a productive infection of oligodendrocytes. Alternatively, the virus is harbored latently within the brain and its expression suppressed by an effective immune response. In fact, B-cells may carry JCV across the blood-brain barrier, where the virus may cross as free virus. JCV may also infect microvascular endothelial cells and thereby cross into the brain [101,212].
So, the primary working hypothesis for development of PML is that at least four events must occur for latent JCV to cause lytic infection of the oligodendrocytes in the brain: (i) the host immune system must be compromised or altered, (ii) the viral NCCR must acquire changes that increase viral transcription and replication in both B-cells and glial cells, (iii) DNA binding factors that bind to recombined NCCR sequence motifs must be present and/or upregulated in infected hematopoietic progenitor, B-cells, and/or glial cells, and (iv) free virus or virus in B-cells must cross the blood-brain barrier and be carried into the brain, where virus is passed to oligodendrocytes and lytic infection takes place. Once the virus is in the brain of the susceptible (immunocompromised) host, PML occurs. These events may occur in the bone marrow, in CD34+ lymphocyte precursors or B-cells in the periphery, or in the brain [119].

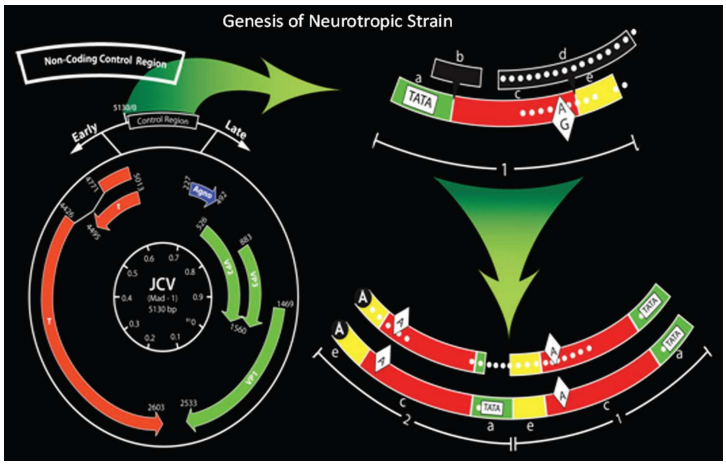


Fig. 14: genesis of the neurotropic strain of JCV (adapted from [185]). The JCV genome is depicted on the left. The archetype virus' NCCR is at the top; at the bottom, is the 98 bp tandem repeat sequence seen in the virus isolated from brains with PML. This is the mutation that must occur.

1.2.3.4.2 Other JCV associated diseases

JCV Granule Cell Neuronopathy (JCV GCN): whereas PML results from JCV infection of glial cells in the brain, JCV granule cell neuronopathy (JCV GCN) is caused by JCV infection of granule cell neurons in the cerebellum. Areas of cell loss in the granule cell layer were first described in up to 5% of PML patients prior to the era of HIV [213].

Granule cells with hypochromatic and enlarged nuclei were also seen in AIDS patients with PML, and JCV DNA was detected by PCR in the cerebellar biopsy of an AIDS patient with cerebellar atrophy [214,215].

This syndrome has now been described by several groups in both HIV-positive and HIV-negative patients, including one with sarcoidosis. JCV GCN can occur in isolation, or concomitantly to PML [reviewed in 107].

Since the granule cell neurons are destroyed by JCV, patients with JCV GCN present with subacute or chronic onset of cerebellar dysfunction, including gait ataxia, dysarthria and incoordination. Magnetic Resonance Imaging (MRI) shows cerebellar atrophy. In addition, cerebellar white matter lesions consistent with PML can also be present. Diagnosis is established by cerebellar biopsy, showing a lytic infection of granule cell neurons by JCV. In the proper clinical and radiological setting, JCV GCN can also be diagnosed by detection of JCV DNA by PCR in the CSF [107].

JCV encephalopathy (JCVE): although JCV mainly infects the brain white matter, gray matter infection can occur.

A case of JCV induced gray matter disease was recently reported, secondary to a productive infection of cortical pyramidal neurons.

The CSF JCV PCR was positive and the diagnosis was confirmed by histological examination which showed fulminant, productive and lytic JCV infection of the cortical pyramidal neurons and astrocytes, associated with laminar necrosis [216].

JCV meningitis: although JCV is not routinely checked when patients present with meningitis, there are several published cases finding JC virus in the CSF of both immunocompromised and immunocompetent patients who presented with meningeal symptoms only. However, the prevalence of detecting JCV in CSF of patients presenting with meningitis has yet to be determined [217].

It is not clear in these cases whether infection was due to JC virus primary infection or reactivation. The presentation of JCV meningitis is consistent with typical meningeal symptoms, with no focal neurological deficits [218]. Unlike PML, there is no focal lesion in the white matter of the brain [219].

Diagnosis is confirmed with detection of JCV in the CSF along with the exclusion of all other neurotropic viruses.

Treatment in JCV GCN, JCVE and JCV meningitis is the same as in classic PML, including HAART in HIV-positive patients, and removal of medications causing immunosuppression in HIV-negative individuals.

1.3 Natalizumab and PML

On 28 February 2005, natalizumab was withdrawn voluntarily from the market after the occurrence of two cases of PML in patients participating in the SENTINEL trial (after 28 and 37 doses of natalizumab, respectively), approximately 1 month before study termination (1,003 of 1,171 MS patients had completed the study); one patient died and the other is still alive [220,221]. These two cases were soon followed by a case of PML in a patient with Crohn's disease treated with natalizumab, who had previously received infliximab, azathioprine and oral corticosteroids (after eight natalizumab infusions) [222], representing an incidence of 1 case of Natalizumab-associated PML in 1,000 patients over a mean treatment period of 17.9 months [223]. Then, the drug was reintroduced in the USA and approved in numerous other countries in June 2006, basing on the notion that benefits for the course of MS outweigh the risks of contracting PML, and after the establishment of an intensive global risk management program. Accumulated safety data from this program have identified longer duration of natalizumab treatment, the use of immunosuppressants before the initiation of natalizumab therapy, and positive status with respect to anti-JC virus antibodies, as assessed with the use of a two-step anti-JC virus antibody assay (STRATIFY JCV, Focus Diagnostics), as risk factors for PML [reviewed in 224].

Since the therapeutic activity of natalizumab in MS is thought to be a result of its ability to prevent activated lymphocytes from entering the CNS, and although natalizumab is not a classical immunosuppressant, the inhibition of leukocyte and monocyte migration may have implications for the immune system surveillance.

The relationship between natalizumab treatment and the appearance of PML is still under investigation, but a number of studies have looked at the specific immune system effects of natalizumab in patients with MS, with a view toward better understanding the potential association between natalizumab treatment and PML. It is clear that treatment with natalizumab significantly suppresses the number of lymphocytes in the CSF [225], and this prolonged decrease of lymphocytes in the CSF may impair immune surveillance in the CNS, leading to JCV reactivation and the development of PML [225-227] (fig. 15).

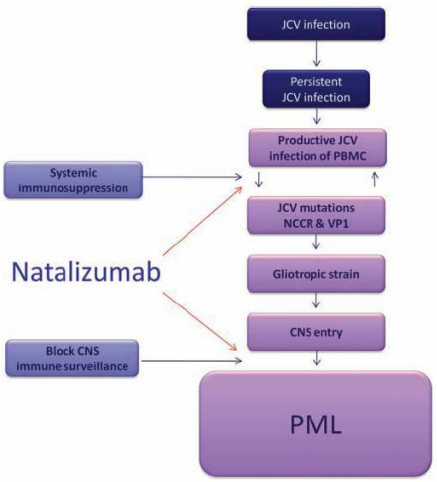


Fig. 15: key speps proposed for the pathogenesis of natalizumab-associated PML. The initial event is primary infection with JCV, which establish a persistent infection. JCV subsequently develops adaptative mutations in the NCCR and VP1 region, allowing the development of pathogenic neurotropic virus with specificity for glial cells. A failure in CNS immune surveillance is required before the patient develops overt PML and natalizumab may lower the threshold for the development of PMLat various levels, as by inhibiting CNS immune surveillance.

There are several differences between natalizumab-associated PML compared with PML in the contest of HIV or malignancy. First, several clinical observations should increase suspicion of natalizumab-associated PML. The most common presenting sympoms are cognitive, motor, language, and visual impairment. Second, although the MRI findings are similar to those of classic PML, the neutralization of the immune system by the medication may lead to very destructive lesions which are rarely seen in other settings. In fact, unlike in classic PML where gadolinium enhancement is usually not seen in presentation, in about one half of natalizumab patients who developed PML there are gadolinium enhancement at diagnosis, indicating ongoing inflammatory host response to JCV. Seizures and paroxysmal events can occur at presentation, which help to differentiate PML from an MS relapse.
The diagnosis of natalizumab-associated PML is established by PCR detection of JCV DNA in the CSF. However, because the viral copy number in the CSF may be very low (500 copies/ml or less), the CSF PCR may be falsely negative, and in several cases of PML brain biopsy may be necessary [228]. This low JCV viral load in the CSF may be explained by the cellular immune response to JCV at baseline observed in MS patients who developed PML after natalizumab treatment, unlike

HIV-1 positive PML patients who have significantly altered cellular immune response [229].
PML associated with natalizumab has a much higher survival rate compared with PML seen in other conditions, possibly because of early diagnosis achieved through clinical vigilance and swift immune reconstitution through natalizumab discontinuation and either plasmapheresis or immunoabsorption. In fact, natalizumab discontinuation is strongly recommended upon the first signs or symptoms and/or MRI findings suggestive of PML [207]. Presently, immune reconstitution, as a restoration of normal immune function, is the only intervention that has demonstrated proven efficacy for PML in both MS and other disease states. Nearly all patients with PML have undergone plasma exchange or immunoadsorption in order to more rapidly remove natalizumab from plasma and to speed up the reconstitution of immune surveillance [230,231].
In considering the treatment of natalizumab-treated patients with MS who develop PML, stopping natalizumab is likely to precipitate IRIS. Indeed, if natalizumab is removed from the circulation by plasma exchange or immunoadsorption, IRIS will occur more rapidly, and PML is controlled more quickly [230].
Predictors of survival in natalizumab-associated PML include younger age at diagnosis, less disability (as measured by Expanded Disability Status Scale [EDSS]) prior to the onset of PML, more localized disease on MRI of the brain, and shorter time from symptoms onset to PML diagnosis [232].

1.3.1 Stratifying risk for natalizumab-associated PML

Key steps in the reduction of risk associated with PML include 1) indentifying patients at increased risk of PML, 2) close monitoring of patients at risk of PML and heightened clinical vigilance for evidence of natalizumab-associated PML, 3) stopping natalizumab where PML is suspected 4) obtaining rapid and accurate diagnosis of PML, and 5) developing strategies for temporary or permanent discontinuation of natalizumab in patients where risk of PML is deemed to be too high [233].
PML cases identified so far, from pre- and post-marketing clinical studies on natalizumab, suggest that the following factors are associated with increased PML risk:

- duration of the treatment
- prior immunosuppressant use
- JCV seropositivity

PML incidence is calculated as the number of PML cases that develop during an interval divided by the number of patients who are ever been exposed to natalizumab for that time of period (either cumulative duration or 12-infusion treatment interval) [234]. The incidence, at present, is determined as 2.75 cases for 1,000 natalizumab-treated MS patients, equal to 298 PML cases for 108,300 natalizumab-treated patients.
The analysis pertaining to the first 79 post-marketing cases (reported up to December 2, 2010) have highlighted as the duration of natalizumab dosing before PML diagnosis ranged from about 1 year to more than 3.5 years (mean 2 years) [reviewed in 235]. Incidence of PML over time was very low in the first 12 months

of treatment but thereafter increased up to 36 months. Thus, over the first two years, the duration of natalizumab exposure clearly relates to the development of PML (fig. 16), and PML risk increases with increasing treatment duration. PML risk may peak after 2 years of treatment or in the third year of therapy, corresponding to 25-36 natalizumab infusions [234]. The number of patients treated for 4 years or more are too small and there are few data beyond this period to allow meaningful calculation of risk for these longer durations of treatment [234].

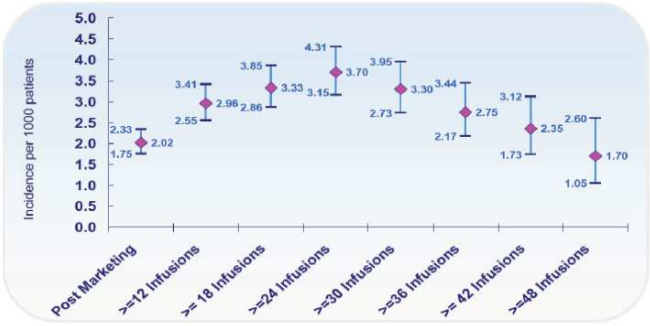


Fig. 16: natalizumab-associated PML incidence by cumulative treatment duration (incidence estimates by treatment duration through 30 November 2011).

After the diagnosis of two early PML cases in combined β-interferon/natalizumab treatment group of SENTINEL trial, it was suggested that the combination may predispose to the development of PML. Patients with MS who developed PML were more likely to have been treated with an immunosuppressant before receiving natalizumab compared with the overall natalizumab-treated population. Immunosuppressant use at any time before initiation of natalizumab treatment confers a 4-fold increased risk of developing PML, despite there being no evidence for residual immune suppression when natalizumab treatment was initiated. The most commonly used immunosuppressive agents in the natalizumab-associated PML patients were mitoxantrone, methotrexate, cyclophosphamide, azathioprine, cladribine, and mycophenolate mofetil (but not β-interferon or GA), but the risk does not appear to be associated with the type of immunosuppressive used, duration of use, or wash-out period between discontinuation of immunosuppressive and initiation of natalizumab [236].
Although it is not clear whether and how the addition of immunomodulatory or immunosuppressant therapy to natalizumab increases the risk of PML, and given the confirmed cases of PML in MS occurred in combination treatment, it seems reasonable that natalizumab should be indicated as monotherapy. In addition, the lack of data to determine whether natalizumab in combination with other drugs is

more effective than natalizumab alone also suggests it would be prudent to use natalizumab only as monotherapy at this time [237].
Assessment of blood samples for the presence of anti-JCV antibodies has been evaluated as another means to identify MS patients at risk of developing PML because JCV infection is required for PML occurrence. JCV specific antibodies can be measured using different assays. Gorelik and colleagues from Biogen Idec developed a 2-step virus-like particle-based enzyme-linked immunosorbent assay (ELISA) (STRATIFY JC virus, 2-step anti-JC virus antibody assay, Biogen Idec, Cambridge, MA) using recombinant VP1 protein to detect JCV specific antibodies, combined with a secondary confirmatory ELISA [239]. This test, which identified 54% of 800 MS patients treated with natalizumab in STRATA study (Safety of Tysabri, redosing and treatment) as seropositive with a 2% annual seroconversion rate, has a false negative rate of 2.5% [238]. In addition, blood from 17 patients who subsequently developed PML were all seropositive when tested with this assay 16-180 months after PML onset, suggesting that seropositivity at baseline is a significant predictive risk factor for PML development.
PML incidence was estimated for seropositive and seronegative patients using the overall PML incidence and anti-JCV antibody prevalence rates for the general MS population (55%) and for the natalizumab-treated PML patients who had pre-PML samples tested for anti-JCV antibodies before PML diagnosis (100%). The incidence of PML in anti-JCV antibody-negative patients and the relative risk of PML between anti-JCV-antibody-positive and negative patients were estimated with sensitivity analysis. In anti-JCV antibody-positive patients, the incidence of PML was estimated to be approximately 2-fold greater than the incidence of the overall natalizumab-treated population. In anti-JCV antibody-negative patients, PML incidence was estimated at 0 cases per 1,000 patients, because no PML patients tested negative for anti-JCV antibodies. Therefore, one hypothetical case of PML was assumed to develop in a patient who tested negative for anti-JCV antibodies, as a part of the sensitivity analysis.
On the basis of this hypothetical case, the estimated incidence of PML in anti-JCV antibody-negative patients would be 0.11 cases per 1,000 patients, or at least 20-fold lower than the incidence in anti-JCV antibody-positive patients [239].
So, the estimated incidence of PML in anti-JCV antibody-positive patients resulted significantly ($p<0.0001$) greater compared with anti-JCV antibody-negative patients. This analysis supports the importance of the use of anti-JCV antibody-serostatus as a risk stratification tool for development of PML.

1.3.2 Quantification of PML risk according to risk factors

Recently, Bloomgren and colleagues developed a risk-factor algorithm to estimate the incidence of PML among patients with and without the established risk factors for natalizumab-associated PML using data from postmarketing sources, clinical studies, and an independent Swedish registry [224]. This algorithm is based on the data of PML risk according to anti-JCV antibodies (positive or negative), prior use of immunosuppressant (yes or no), and duration of natalizumab treatment (1 to 24 months versus 25 to 48 months).
They determined the number of PML cases among patients who had received 1 to 24 months of natalizumab treatment or at least 25 to 48 months of Natalizumab

treatment, stratified according to status with respect to prior use of immunosuppressants. Then, considering two risk-factors, they estimated the incidence of PML among patients who were positive for anti-JCV antibodies by dividing the number of patients with natalizumab-associated PML who were positive for anti-JCV antibodies by estimated number of natalizumab-treated patients who were positive for anti-JCV antibodies in the postmarketing setting [224] (fig. 17).

Since exposure to JCV is fundamental for the development of PML, patients who were negative for anti-JCV antibodies represent the subgroup at the lowest risk in the PML risk-stratification algorithm, with an estimated incidence of 0.09 cases or fewer per 1,000 patients. In the highest-risk subgroup (patients who have all the three risk-factors), the estimated incidence of PML was approximately 11.1 cases per 1,000 patients [224,240].

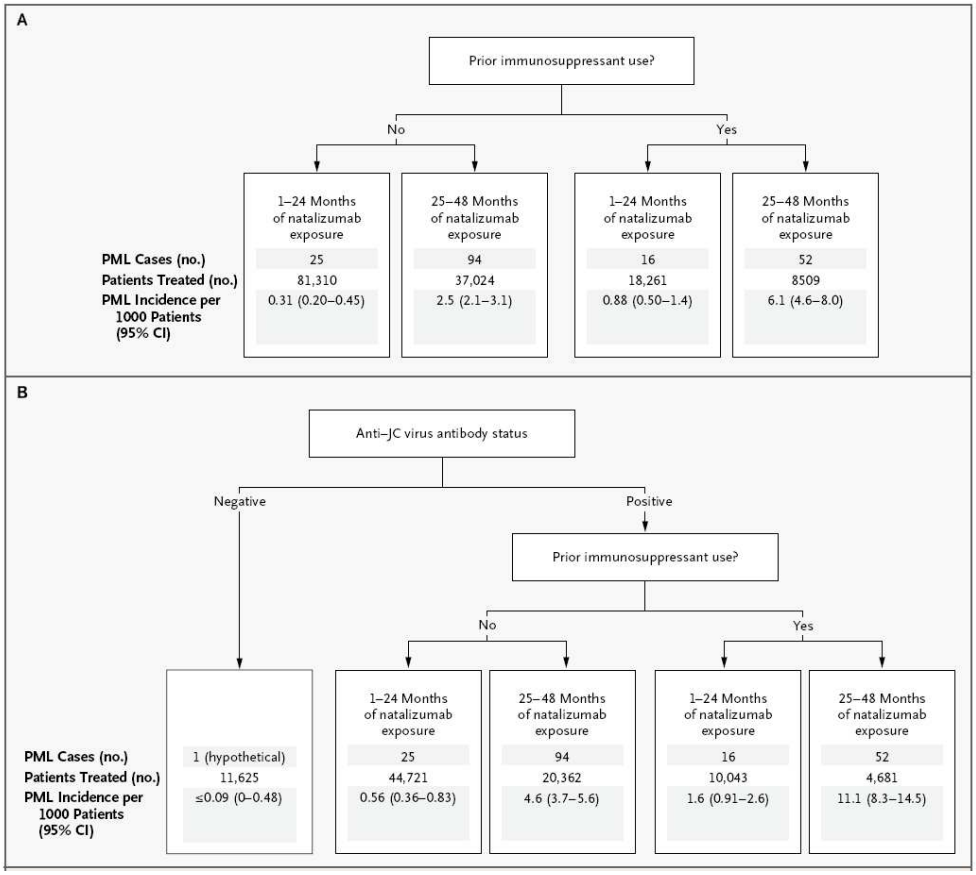


Fig. 17: approximate incidence of PML, stratified according to risk factors (from [224]) calculated through February, 2012). Estimates of the incidence of PML are shown, stratified according to prior or no prior use of immunosuppressants and duration of natalizumab treatment (Panel A) and according to positive or negative status with respect to anti-JC virus antibodies, prior or no prior use of immunosuppressants, and duration of natalizumab treatment (Panel B).

1.3.3 Management of PML risk

For patients with MS who are considering or receiving natalizumab therapy, the risk of PML can be stratified according to positive or negative status with respect to anti-JCV antibodies, prior or no prior use of immunosuppressants, and the duration of natalizumab treatment. This may help in the identification of patients for whom natalizumab therapy is most appropriate and may reduce the incidence of PML, a serious potential complication of the therapy.

Nevertheless, clinical vigilance for the clinical features of PML remains of critical importance for PML risk management strategy. All patients undergoing natalizumab therapy must have a baseline MRI scan within 3 months before starting treatment, and annually during the therapy. Moreover, all anti-JCV antibody-negative patients may potentially be treated with minimal risk, but it is currently recommended that negative anti-JCV antibody status should be re-evaluated on a six-month basis. Once a patients tests positive for anti-JCV antibodies, he should be considered exposed to the virus, with regards to risk stratification, and no further testing is recommended. On suspicion of PML, natalizumab should be stopped, and MRI and CSF examination undertaken (fig. 18).

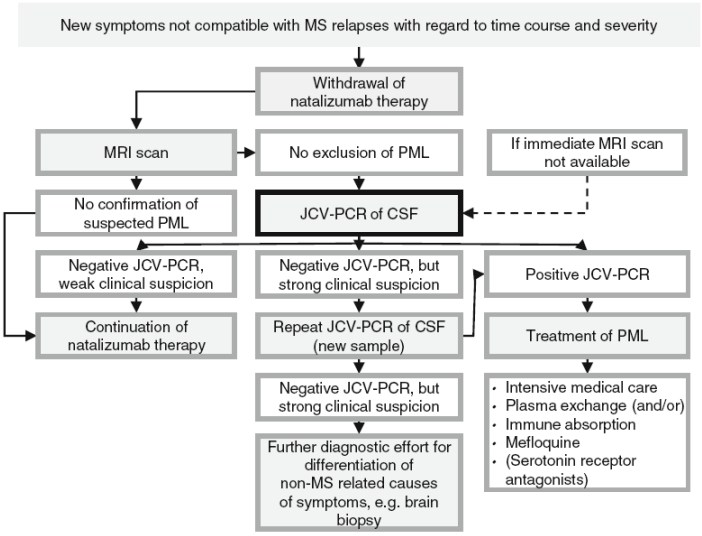


Fig. 18: algorithm for diagnosis and treatment of natalizumab-associated PML.

1.3.4 Identification of markers for natalizumab-associated PML development and related studies

A laboratory marker that predicts the likelihood of PML development is required for selection of people most likely to benefit safely from natalizumab treatment and for early detection of PML.

Because PML is caused by JCV, and detection of JCV is a strong indicator of viral presence, the presence of JCV DNA in peripheral body fluids may be useful for stratification of PML risk. Nevertheless, there is no convincing evidence that

monitoring JCV replication in either blood (plasma or PBMCs), urine or CSF provides useful information about the risk of developing PML.

Chen and colleagues [229] has suggested that subclinical reactivation of JCV occurs frequently in natalizumab treated patients and that JCV shedding, identified in urine samples, is associated with a transient drop in the JCV-specific cellular immune response. In particular, they followed 19 patients with RR-MS, enrolled in the study before they started natalizumab treatment over an 18-month period. Blood and urine samples were collected before starting the therapy and then at 3, 6, 9, 12, and 18 months, at the time of their natalizumab infusion. Quantitative real-time polymerase chain reaction (Q-PCR) was used for the detection of JCV DNA. They showed an increase in the frequency of JCV excretion in the urine, which was detected in 63% of the 19 patients after 12 months of natalizumab therapy, and a detection of viremia in 60% of the patients tested at 18 months.

This has not been replicated in larger studies [240-243], as in the analysis performed on urine and blood samples from 1,400 individuals participating in clinical studies on natalizumab, in order to determine if the presence of JCV DNA could be useful in predicting PML risk in natalizumab-treated MS patients [241]. Using Q-PCR it was reported that viremia was rare, treatment with natalizumab was not associated with an increased prevalence of JCV viremia at the time points that samples were taken, the presence of JCV DNA in the blood was not associated with the development of PML, and patients who developed natalizumab-related PML tested negative in blood and PBMCs prior to the onset of sympthoms [241].

Another study from Sadiq and colleagues reported the detection of JCV DNA by PCR in the CSF of 8/200 MS patients receiving natalizumab. Subsequent PCR in these 8 patients was negative after stopping natalizumab. None of the patients in this study developed PML [244].

Thus, there is no evidence that, using currently available methods, testing for JCV DNA in biological fluids is useful for predicting DNA development. In contrast, serological assessment of anti-JCV antibody status may offer a more sensitive and practical means of identifying patients who may be infected by JCV.

However, a recent study from Laroni and colleagues has evaluated the utility of testing JCV DNA, together with anti-JCV antibodies, in biological samples of natalizumab-treated patients, as a tool for PML risk stratification [245]. 126 subjects were enrolled in this study and a cross-sectional study was performed on 63 patients testing JCV-DNA in blood, PBMCs, and urine. The presence of JCV-DNA was assessed longitudinally in a cohort of 33 subjects, one of which developed PML, and serum samples from another patients who developed PML was tested retrospectively. Anti-JCV antibodies and urinary JCV-DNA were both tested in 73 patients. The cross-sectional and longitudinal studies reported that no reactivation of JCV DNA in urine or other biological samples occurs during natalizumab treatment. The patient who developed PML during the longitudinal study was constantly positive for JCV DNA in urine samples since before starting the therapy. This observation supports the hypothesis that PML in natalizumab-treated patients is likely to be a reactivation of a precedent infection, rather than a first infection.

Regarding the analysis on the correlation between urinary JCV DNA and anti-JCV antibodies, the anti-JCV antibodies test confirmed its higher sensibility (52% positive patients) compared to urinary JCV DNA (42.5%), with a false negative rate of about 2.5%, probably due to an inter-individual difference in the immune response to JCV infections. The authors of this work have concluded that testing JCV DNA in serum, whole blood or PBMCs has no clinical utility, while testing urinary JCV DNA allows identifying patients who harbor the JCV. Thus, this could be useful for identifying individuals at risk of PML among those who resulted negative at anti-JCV antibodies test.

2. Aim of the study

MS is a complex chronic immune mediated disease of the CNS. Its etiology is currently unknown and its pathogenesis is only partially understood. Complex genetic traits as well as environmental factors determine the susceptibility to develop the disease and also immune mechanisms play an essential role in driving the disease process.

In addition to the immunomodulatory therapies approved for MS, mAbs have emerged as promising treatments to reduce the symptoms of MS. In particular, the humanized mAb natalizumab binds and block the α4 integrin, preventing the binding of T-lymphocytes to the adhesion molecules VCAM-1 on endothelial cells, their migration across the BBB and the consequent inflammation. Although its proven efficacy in RR-MS treatment, natalizumab therapy results in reduced immune surveillance of the CNS that predisposes to the reactivation of latent viruses, such as JCV, the etiologic agent of PML.

By October 2012, a total of 298 cases of PML have been reported for 108,300 natalizumab-treated MS patients. Sixthy-three out of 298 died, representing a death rate of 21%.

The main aim of this work is to better understand the molecular basis of demyelinating diseases, by studying the association between natalizumab therapy, JCV reactivation and PML, and to identify possible biomarkers associated with an elevated risk of developing PML in natalizumab-treated MS patients, in order to further stratify patients at risk. In addition, herpes viruses Epstein-Barr (EBV), Varicella Zoster (VZV), Human Cytomegalovirus (HCMV), Herpes Simplex-1 (HSV-1) and Human Herpes Virus-6 (HHV-6) have been searched to evaluate if they could be involved in demyelinating diseases pathogenesis. These objectives have been achieved through an extensive longitudinal study involving three different demyelinating diseases: MS, PML and NDLE, a PML-like leukoencephalopathy, which occurs in the absence of the JCV genome in the CSF.

A total of 121 patients was enrolled in this study and their biological fluids collected at various stages of the diseases, in order to evaluate JCV reactivation/replication and its relation with natalizumab treatment and PML risk.

3. Materials and Methods

3.1 Biological samples

CSF, peripheral blood (PB), serum and urine samples were collected, processed and freezed until the time of use.
In particular, CSF and urine samples were aliquoted and stored at -80°C until the time of DNA extraction, while PB samples were taken in sterile tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA), aliquoted, and stored at 4°C until the time of DNA extraction, than at -80°C.
Sera samples were collected in tubes containing an inert barrier gel that is present in the bottom of the tube. The samples were centrifuged at 3000 rpm for 10 minutes, and during this time the barrier gel moves upward to the serum-clot interface, where it forms a stable barrier separating the serum from fibrin and cells. The serum is then aliquoted and stored at -80°C before the DNA extraction.

3.2 DNA extraction

DNA extraction from biological samples has been carried out with commercial kits, following the manufacturer instructions.
In particular, the general principles of the protocols are lysis buffers, that destabilizes the cell membranes, leading to the breakdown of cellular structure. The addition of a chaotropic salt during or after cell lysis, disrupts the protein structure by interfering with hydrogen bonding, Van der Waals interactions, and the hydrophobic interactions. Cellular proteins are largely insoluble in the presence of the chaotropic agent and can be removed by centrifugation or filtration. This DNA extraction methods are based on the binding properties of silica or glass particles. DNA will bind to silica particles with a high affinity in the presence of a chaotropic salt. After the other cellular components have been removed the DNA can be released from the silica/glass particles by suspending them in water. Without the chaotropic salt the DNA no longer binds to the silica/glass and is released into solution.

3.2.1 DNA extraction from CSF, urine and serum samples

Viral DNA extraction from CSF, urine and serum samples is performed with NucleoSpin RNA Virus kit (Macherey Nagel, Germany), which can be applied to acellular bodily fluids. With this commercial kit, viruses are lysed quickly and efficiently by Lysis Buffer RAV1 which is a highly concentrated solution of guanidinium thiocyanate. DNA viruses are usually difficult to lyse and require Proteinase K digestion. Lysis buffer and ethanol create appropriate conditions for binding of nucleic acids to the silica membrane of the columns.
Contaminations (potential PCR inhibitors) like salts, metabolites and soluble macromolecular cellular components are removed in simple washing steps with ethanolic buffers RAW and RAV3. The nucleic acids can be eluted in water and are ready-for-use in subsequent reactions.
In particular, the exact procedure for viral DNA extraction from urine and sera samples is based on the addition of 600 µl lysis buffer RAV1 to 150 µl of the sample and 20 µl of proteinase K solution (20 mg/ml stock solution). The mixture is incubated for 5 minutes (min) at 70°C for sample lysis. Then, 600 µl ethanol (96–

100 %) are added to the clear lysis solution and the lysed sample is loaded in the columns and centrifuged for 1 min at 8,000 x g. Two washing steps are carried out with 500 µl of buffer RAW and 600 µl of buffer RAV3, separated by centrifugation for 1 min at 8,000 x g. This washing step removes contaminants and PCR inhibitors. The third washing step is performed adding 200 µL buffer RAV3, and centrifuging for 4 min at 11,000 x g to remove ethanolic buffer RAV3 completely. Since residual buffer RAV3 may inhibit subsequent reactions which are extremely ethanol-sensitive, the column is incubated for 1 min at 70°C to remove any remaining traces of ethanol. To elute viral DNA, 50 µl RNase-free H2O (preheated to 70°C) is added and the column is incubate for 2 min at 70°C, and then centrifuged for 1 min at 11,000 x *g*.

3.2.2 DNA extraction from PB samples

The QIAamp DNA Blood Mini Kit (Qiagen, USA) simplifies isolation of DNA from blood with fast spin-column. DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure nucleic acid to be eluted in water.
The procedure is based on the addition of 40 µl of Qiagen protease (for proteolysis) to 400 µl of whole blood and 400 µl of lysis buffer AL. The mixture is incubated at 56°C for 10 min. Then, after the addition of 400 µl of ethanol (96-100%), the mixture is applied on the QIAamp mini spin column and centrifuged for 1 min at 6,000 x g, in order to allow DNA adsorption onto the silica membrane. DNA bound to the membrane is washed in two centrifugations, and the use of two different wash buffers, buffer AW1 and buffer AW2, significantly improves the purity of the eluted DNA. In addition, wash conditions ensure complete removal of residual contaminants without affecting DNA binding. The washing steps are achieved with 500 µl buffer AW1, followed by a centrifugation for 1 min at 6,000 x g, and 500 µl buffer AW2, followed by a centrifugation for 3 min at full speed (20,000 x g). Further centrifuge at full speed for 1 minute helps to eliminate the chance of residual buffer AW2. For DNA elution, 50 µl distilled water are added to the column, which is then incubated at room temperature for 5 min for yields improvement, and centrifuged at 6000 x g for 1 min.
Thus, the concentration of the eluted DNA is quantified at a wave length of 260 nm by means of a spectrophotometric analysis with the SmartSpec Plus Spectrophotometer (Bio-Rad), using a 50-fold dilution.

3.2.3 RNA extraction from CSF samples

HIV-1 RNA is purified from CSF samples by means of QIAamp viral RNA mini kit. The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the membrane, and the sample is loaded onto the column. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers. High-quality RNA is eluted in water. The exact procedure is based on the addition of 560 µl of lysis buffer AVL containing carrier RNA to 140 µl of CSF. After an incubation at room temperature for 10 min to allow the lysis of viral particles, the mixture is

added of 560 µl of ethanol (96-100%). This mixture is applied to the column, which is centrifuged for 1 minute at 6,000 x g. The membrane in the column is washed with 500 µl of buffer AW1, with a centrifugation for 1 min at 6,000 g. The second washing is performed with 500 µl of buffer AW2 and is followed by a centrifugation for 3 min at 20,000 g (full speed). To eliminate any chance of possible buffer AW2 carryover, the column is further centrifuged for 1 min at full speed. Viral RNA is eluted in 50 µl of RNase-free water, after incubation at room temperature for 1 min and a centrifugation for 1 min at 6,000 x g.

3.3 Quantitative Real-Time PCR

Real-Time PCR, also called quantitative PCR (Q-PCR), is one of the most powerful and sensitive gene analysis techniques available and is used for a broad range of applications including quantitative gene expression analysis, genotyping, SNP analysis, pathogen detection, drug target validation and for measuring RNA interference. Since, it measures PCR amplification as it occurs, it represents an evolution of the traditional standard PCR, in which results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid.
In particular, Q-PCR systems use fluorescent reporter dyes to combine DNA amplification and detection steps in a single tube format, and measure products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR itself. Regarding the fluorescent reporter, its signal increases in direct proportion to the amount of PCR product.
The Q-PCR assays of this study were performed using the TaqMan chemistry, based on fluorogenic-labeled probes that use the 5' nuclease activity of Taq DNA polymerase, the absolute quantitation method, and the 7500 RealTime PCR System (Applied Biosystem, USA).

3.3.1 Q-PCR for HIV-1

To detect the presence of HIV-1 genome in the CSF, a Q-PCR targeting the gag gene was performed using primers and probe shown in tables 4 and 5. The probe was labeled at the 5' end with the reporter dye FAM and at the 3' end with the quencher dye MinorGrooveBinder (MGB). The standard curve was obtained using the OptiQuant HIV-1 RNA Quantification panel (Acrometrix, USA), containing seven members, one of which was the negative. Each panel member contained HIV-1 viral RNA at a predetermined level and in particular intact and incapsidated viral particles which have been subjected to an extraction step for viral RNA releasing. The positive panel members were produced by making quantitative dilutions.
The limit of detection of this assay was 1 copy/reaction.

Table 4: probe used in the Q-PCR assay for HIV-1.

PROBE	POSITION	SEQUENCE
HIVprobeMGB	488-507	5‘- 6-FAM- TGT TAA AAG AGA CCA TCA AT- MGB- 3‘

Table 5: primers used in the Q-PCR assay for HIV-1.

PRIMER	POSITION	SEQUENCE
HIVMGBF	467-486	5’- GAC ACT AAG CAG CCA TGC AA -3‘
HIVMGBR	509-531	5’- CTA TCC CAT TCT GCA GCT TCC T -3‘

The assay is performed using The TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, USA), which is designed for the reverse transcription (RT) and PCR amplification of a specific target RNA from either total RNA or mRNA. One-step RT-PCR performs RT as well as PCR in a single buffer system without interruption.

The reaction mix was prepared as follows:

- Taqman Universal PCR Master Mix (2X) 25 µl
- MultiScribe (40X) and RNase inhibitor mix 1.25 µl
- HIVMGBF (0.9 µM) 0.7 µl
- HIVMGBR (0.9 µM) 0.7 µl
- HIVprobeMGB (0.2 µM) 0.1 µl
- Template RNA 10 µl
- H_2O up to 50 µl

The thermal cycling parameters are the following:

	RT	AmpliTaq Gold activation	PCR	
	Hold	Hold	Cycles (40 cycles)	
			Denature	Anneal/extend
Time	30 min	10 min	15	1 min
Temperature	48°C	95°C	95°C	60°C

3.3.2 Q-PCR for JCV

The Q-PCR protocol for the detection of JCV DNA in CSF, urine, PB and sera samples has been already described elsewhere [246]. The test sensitivity was 2 copies/reaction.
The standard curve for JCV assay was constructed with ten-fold serial dilutions (range: $1.1x10^4$ to 1,1x10 plasmid copies/µl) of a plasmid containing the entire JCV genome, MAD1 strain (Advanced Biotechnologies, USA).
Primers and probe for this assay, targeting the T-Ag region, are described in table 6 and 7:

Table 6: probe used in the Q-PCR assay for JCV.

PROBE	POSITION	SEQUENCE
JCV Probe	4323-4350	5‘- 6-FAM- TCA TCA CTG GCA AAC ATT TCT TCA TGG C-TAMRA -3‘

Table 7: primers used in the Q-PCR assay for JCV.

PRIMER	POSITION	SEQUENCE
JCV Forward	4299-4321	5’- GAG TGT TGG GAT CCT GTG TTT TC -3‘
JCV Reverse	4352-4375	5’- GAG AAG TGG GAT GAA GAC CTG TTT -3‘

The reaction mix was prepared as follows:

- Maxima probe qPCR Master Mix (2x) (M Medical) 12.5 µl
- JCV Forward (0.4 µM) 0.12 µl
- JCV Reverse (0.4 µM) 0.12 µl
- JCV Probe (0.2 µM) 0.05 µl
- Template DNA 5 µl
- H_2O up to 25 µl

The thermal cycling parameters are the following:

	Incubation	AmpliTaq Gold activation	PCR	
			Cycles (40 cycles)	
			Denature	Anneal/extend
Time	2 min	10 min	15	1 min
Temperature	50°C	95°C	92°C	60°C

3.3.3 Q-PCR for herpes viruses

Q-PCR assays were performed for the detection of herpes viruses EBV, VZV, CMV, HSV-1 and HHV-6, using primers and probes described in the table 8 and 9:

Table 8: probe used in the Q-PCR assay for Herpes viruses.

	PROBE	POSITION	SEQUENCE
EBV	EBV Probe	Unknown	Sequence proprietary (Applied Biosystem, USA)
VZV	VZV Probe	58286-58307	5‘- 6-FAM- ACC AGA TCC CGA CGA AGC GTG C-MGB -3‘
HCMV	CMV Probe	Unknown	Sequence proprietary (Applied Biosystem, USA)
HSV-1	HSV1 Probe	Unknown	Sequence proprietary (Applied Biosystem, USA)
HHV-6	HHV6 Probe	Unknown	Sequence proprietary (Applied Biosystem, USA)

Table 9: primers used in the Q-PCR assay for Herpes viruses.

	PRIMER	POSITION	SEQUENCE
EBV	EBV Forward	Unknown	Sequence proprietary (Applied Biosystem, USA)
	EBV Reverse		
VZV	VZV Forward	58264-58284	5‘- ACA AAA ACA CCC GAC TCG AAA -3‘
	VZV Reverse	58309-58330	5’- TCT ATT GGC ACG CAA CTC AAC T -3’
HCMV	CMV Forward	Unknown	Sequence proprietary (Applied Biosystem, USA)
	CMV Reverse		
HSV-1	HSV1 Forward	Unknown	Sequence proprietary (Applied Biosystem, USA)
	HSV1 Reverse		
HHV-6	HHV6 Forward	Unknown	Sequence proprietary (Applied Biosystem, USA)
	HHV6 Reverse		

The reaction mixes were prepared as follows:

- Maxima probe qPCR Master Mix (2x) (M Medical) 12.5 µl
- Primer Forward (0.4 µM/ 0.9 µM only for HHV-6) 0.12 µl/ 0.27 µl
- Primer Reverse (0.4 µM/ 0.2 µM only for HHV-6) 0.12 µl/ 0.06 µl
- Probe (0.2 µM) 0.05 µl
- Template DNA 5 µl
- H_2O up to 25 µl

The thermal cycling parameters are the following:

	Incubation	AmpliTaq Gold activation	PCR	
			Cycles (40 cycles)	
			Denature	Anneal/extend
Time	2 min	10 min	15	1 min
Temperature	50°C	95°C	92°C	60°C

Stardard curves for the quantyification of the viral genomes were constructed using ten-fold serial dilutions of plasmids containing herpes viruses genome, and in particular:

- EBV: B95-8 strain (dilution range: $1.2x10^4$ to 1.2x10 copies/µl)
- VZV: Rod strain (dilution range: $1.5x10^4$ to 1.5x10 copies/µl)
- HCMV: AD169 strain (dilution range: $1.6x10^4$ to 1.6x10 copies/µl)
- HSV-1: MacIntyre strain (dilution range: $1.4x10^4$ to 1.4x10 copies/µl)

- HHV-6: GS strain (dilution range: $1.9x10^4$ to 1.9x10 copies/µl)

The viral copy concentration for all the Q-PCR assays was log-transformed and expressed as log [copies/CSF ml] for CSF, sera, and urine samples, and as log [copies/µg of isolated DNA] for the PB samples.

3.4 Standard PCR

PCR was applied to the molecular analysis of JCV strains amplified by Q-PCR in CSF and urine samples. All the amplifications were performed by using the 2720 Termal Cycler (Applied Biosystem, USA). PCR products were then loaded on agarose gels and analyzed bt electrophoresis using Tris-Borate-EDTA (TBE) buffer. The DNA fragments were visualized with ethidium bromide (1µg/ml) under the UV light of the gelDoc-it Image System (UVP, USA).

3.4.1 PCR for JCV VP1 gene

For JCV genotyping, a 215 bp fragment of the JCV VP1 gene was amplified using a single set of primers, JLP15 (forward primer) and JLP16 (reverse primer) [247]. The exact primer sequences and the position on JCV genome are indicated in table 10:

Table 10: probe used in the PCR assay for JCV VP1 gene.

PRIMER	POSITION	SEQUENCE
JLP15	1710-1734	5'- ACA GTG TGG CCA GAA TTC CAC TAC C -3'
JLP16	1902-1924	5'- TAA AGC CTC CCC CCC AAC AGA AA -3'

The reaction mix was prepared as follows, using reagents from EuroClone, Italy:

- 10X Buffer (without Mg^{2+}) 5 µl
- dNTPs (10mM) 2 µl
- Mg^{2+} (50 mM) 2 µl
- JLP15 (10 pmol/µl) 1.5 µl
- JLP16 (10 pmol/µl) 1.5 µl
- EuroTaq DNA Polymerase (5U/µl) 0.4 µl
- Template DNA 5 µl
- H_2O up to 50 µl

The amplification program is the following:

STEP	TIME	TEMPERATURE	NO. OF CYCLES
Initial denaturation	9 min	94°C	1
Denaturation	30 sec	94°C	48
Annealing	30 sec	63°C	
Extension	30 sec	72°C	
Final extension	10 min	72°C	1

3.4.2 Nested PCR for JCV TCR

The analysis of the TCR region and its rearrangements was performed using a protocol, previously reported [246], for a nested PCR, which involves two sets of primers, used in two successive runs of PCR. The second pair of primers (nested primers) for the second PCR bind within the first PCR product and produce a second PCR product that is shorter than the first one. This approach has greater specificity than regular PCR, and it can yield detectable product, otherwise not visible in a simple PCR.
Primers JRE1 and LP2 (outer) and RFOR and RREV (inner) are used to amplify a 353 bp fragment belonging to the JCV TCR.
The exact primer sequences and the position on JCV genome are indicated in table 11.

Table 11: primers used in the PCR assay for JCV TCR.

PRIMER		POSITION	SEQUENCE
OUTER	JRE1	4989-5009	5'- CCT CCC TAT TCA GCA CTT TGT -3'
	LP2	518-537	5'- TGC GGC ACC CAT GAA CCT GA -3'
INNER	RFOR	5085-5104	5'- CAG AAG CCT TAG CGT GAC AGC -3'
	RREV	291-310	5'- GCC TCC ACG CCC TTA CTA CT -3'

The reaction mix for the first amplification (outer) was prepared as follows, using reagents from EuroClone, Italy:

- 10X Buffer (without Mg^{2+}) 5 µl
- dNTPs (10mM) 2 µl
- Mg^{2+} (50 mM) 2 µl
- JRE1 (10 pmol/µl) 2 µl
- LP2 (10 pmol/µl) 2 µl
- EuroTaq DNA Polymerase (5U/µl) 0.4 µl
- Template DNA 5 µl
- H_2O up to 50 µl

The first amplification program, which yielded a 678 bp product, is the following:

STEP	TIME	TEMPERATURE	NO. OF CYCLES
Initial denaturation	3 min	95°C	1
Denaturation	30 sec	94°C	30
Annealing	30 sec	59°C	
Extension	30 sec	72°C	
Final extension	7 min	72°C	1

The reaction mix for the second amplification (inner) was prepared as follows, using reagents from EuroClone, Italy:

- 10X Buffer (without Mg^{2+}) 5 µl
- dNTPs (10mM) 2 µl
- Mg^{2+} (50 mM) 2 µl
- RFOR (10 pmol/µl) 2 µl
- RREV (10 pmol/µl) 2 µl
- EuroTaq DNA Polymerase (5U/µl) 0.4 µl
- Outer amplified product 5 µl
- H_2O up to 50 µl

The second amplification program, which yielded a 353 bp product inside the former, is the following:

STEP	TIME	TEMPERATURE	NO. OF CYCLES
Initial denaturation	3 min	95°C	1
Denaturation	30 sec	94°C	30
Annealing	30 sec	63°C	
Extension	30 sec	72°C	
Final extension	7 min	72°C	1

3.5 DNA sequencing

Each PCR fragment was sent to an external facility (Primm Srl, Italy) for the direct sequencing of both viral strands, on the basis of Sanger sequencing principles. Sequence homology searches were performed using a nucleotide BLAST search, database set “others”, from NCBI site (http://blast.ncbi.nlm.nih.gov/Blast.cgi), according to Agostini and colleagues [248] for JCV genotyping, and Ault and colleagues [249] and Jensen and Major [186] for JCV TCR rearrangements.

3.6 Anti-JCV antibodies test

All natalizumab-treated RR-MS pateints underwent the compulsory 2-step virus-like particle-based ELISA, used to detect anti-JCV antibodies in human sera and plasma. The assay incorporates an ELISA test and a supplemental confirmation test. The presence or absence of anti-JCV antibodies is determined spectrophotometrically at 450 nm. In the supplemetal confirmation test, soluble MAD-1 JCV virus-like particles (VLPs) were used to pre-adsorb antibodies against JCV prior to evuation of samples in the ELISA, in order to enhance binding of both low- and high-affinity antibodies specific for JCV, thereby increasing the sensitivity of the test. Results were calculated as percentage inhibition to determine decreases in reactivity in the ELISA after the samples were pre-adsorbed with JCV VLPs. Since this ELISA test is characterized by a false-negative rate of 2.5%, from March, 2012 it has been replaced by a second generation test, called STRATIFY JCV DxSelect test, characterized by an increased sensitivity.

3.7 Statistical analysis

Distribution data resulting from the Q-PCR analysis of all biological samples from the patients enrolled in this study were analyzed using the Student’s t-test.
A chi-square test was used to analyze the statistical significance between a categorical outcome and a categorical determining variable.
Only correlation with a $p<0.05$ were considered statistically significant.

4. Results

Results regarding the virological screening and molecular characterization of amplified JCV strain from PML and NDLE patients, which belong to a broadest study performed in our laboratory, are described in the section Results I. These data have been already reported in the literature, in the Journal of Cellular Physiology [250].
Results regarding the longitudinal study on natalizumab-treated RR-MS patients are shown in section Results II.

4.1 Results I

4.1.1 Case study

From 2007, twenty-two HIV-1 positive (HIV-1+) patients with a confirmed molecular diagnosis of PML were recruited at the Infectious Diseases Department of San Matteo Hospital, Pavia and at the Neurological Department of the Mondino Institute, Pavia, for a longitudinal study.
For these PML patients, on the basis of the presence/severity of symptoms at the onset and during the clinical course of the disease, three different groups were defined:

1. 10 PML patients with a multisymptomatic onset , typical for PML, and a classical disease course, who died within one year after the time of the diagnosis (CPML);
2. 9 PML patients who had a typical multisymptomatic onset, but a benign disease course (benign PML);
3. 3 asymptomatic PML patients, showing brain lesions typical of PML at MRI, and JCV DNA in the CSF.

CSF, PB and urine samples were collected at the time of the diagnosis and after 6 months, when possible, to monitor the clinical course of the disease. The immune and virological features (CD4+ cells count and HIV-1 plasma viral load) were acquired for each patient from clinical recors.
In addition, 6 HIV-1 negative patients with a confirmed molecular diagnosis of PML were included in this study, in order to analyse and compare the CSF JCV load and its molecular characterization at the time of the enrolment.
These HIV-1 negative PML patients, who were not subjected to follow-up analysis, were affected with different form of haematological malignancy, in particular 4 cases of chronic lymphocytic leukemia (CLL) and 2 cases of non-Hodgkin's lymphoma. Two patients with CLL and one patients with non-Hodgkin's lymphoma died within a few months from disease onset, two others are still alive, but with severe neurological conditions, and the remaining patient was unable to be contacted for follow-up.
Twenty-two HIV-1+ patients with NDLE were also selected at the Clinic of Infectious Diseases, San Matteo Hospital, Pavia, and underwent virological analysis on CSF at the time of leukoencephalopathy diagnosis.

4.1.2 Demographic and virological features of PML patients at baseline

The demographic and virological findings at baseline are shown in table 12.
The twenty-two HIV-1+ PML patients included 21 males and 7 female, with a mean age of 42 years (range: 20-70 years) and all but one were on HAART therapy at the time of PML diagnosis. The mean baseline CD4+ cells count was 70.9±29.8/µl for the CPML patients, 220.2±78.4/µl for the benign Pml patients and 424±157.9/µl for the asymptomatic PML patients (CPML vs. benign and vs. asymptomatic PML: $p<0.05$) (fig. 19). The mean baseline HIV-1 load was log 4.3±4.1 copies/ml, log 3.3±3.2 copies/ml and log 4.7±4.6 copies/ml for the CPML, benign PML and asymptomatic PML patients, respectively.
At the time of the enrolment, the mean JCV load in the CSF determined by means of Q-PCR was log 6.0±1.2 copies/ml for the CPML patients, log 4.0±1.0 copies/ml for the benign PML patients, log 4.2±0.5 copies/ml for the asymptomatic PML patients, and log 5.8±1.3 copies/ml for the HIV-1 negative PML patients (CPML vs. benign PML: $p<0.01$; CPML vs. asymptomatic PML: $p<0.05$; HIV-1 negative vs. benign PML: $p<0.01$) (fig. 20). The mean HIV-1 RNA load in the CSF was log 2.7±1.3 copies/ml for the CPML patients, log 2.2±1.0 copies/ml for the benign PML patients and log2.7±1.7 copies/ml for the asymptomatic PML patients.
JCV DNA was detected from the PB in 4 of 7 samples from CPML patients (log 3.15±1.1 copies/µg), in 1 of 7 samples from the benign PML patients and in none of the samples from the asymptomatic PML patients. JCV DNA was also amplified in 4 out of 7 urine samples from CPML patients (log 6.6±1.2 copies/ml), in none of the samples from the benign PML patients and in 1 sample from the asymptomatic PML patients.
Regarding the herpes viruses analysis on PML CSF samples at baseline, no herpes virus genomes were amplified in PML patients.

Table 12: demographic and virological findings at baseline.

	HIV-1+ PML PATIENTS			HIV-1 NEGATIVE PML PATIENTS
	CPML (10 patients)	**BENIGN PML (9 patients)**	**ASYMPTOMATIC PML (3 patients)**	**HIV-1 NEGATIVE PML (6 patients)**
Male/Female	9/1	5/4	3/0	5/1
Mean CD4+ cells count	70.9±29.8/μl[a,b]	220.2±78.4/μl[a]	424±157.9/μl[b]	n.a.
Mean HIV-1 viremia	log 4.3±4.1 copies/ml	log 3.3±3.2 copies/ml	log 4.7±4.6 copies/ml	n.a.
Mean CSF JCV load	log 6.0±1.2 copies/ml*,**	log 4.0±1.0 copies/ml*,***	log 4.2±0.5 copies/ml**	log 5.8±1.3 copies/ml***
Mean CSF HIV-1 load	log 2.7±1.3 copies/ml	log 2.2±1.0 copies/ml	log 2.7±1.7 copies/ml	n.a.
JCV DNA in PB	log 3.15±1.1 copies/ml	log 2.0 copies/ml	0	n.a.
JCV DNA in urine	log 6.6±1.2 copies/ml	0	log 5.3 copies/ml	n.a.

([a]CPML vs. benign PML: p<0.05, [b]CPML vs. asymptomatic PML: p<0.05; *CPML vs. benign PML: p<0.01;** CPML vs. asymptomatic PML: p<0.05;*** HIV-1 negative vs. benign PML: p<0.01)

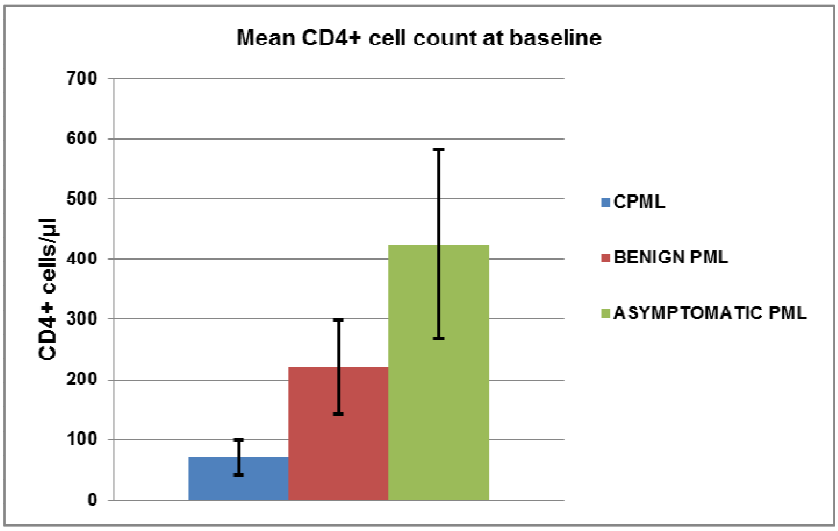


Fig. 19: mean CD4+ cell count for HIV-1+ PML patients at baseline.

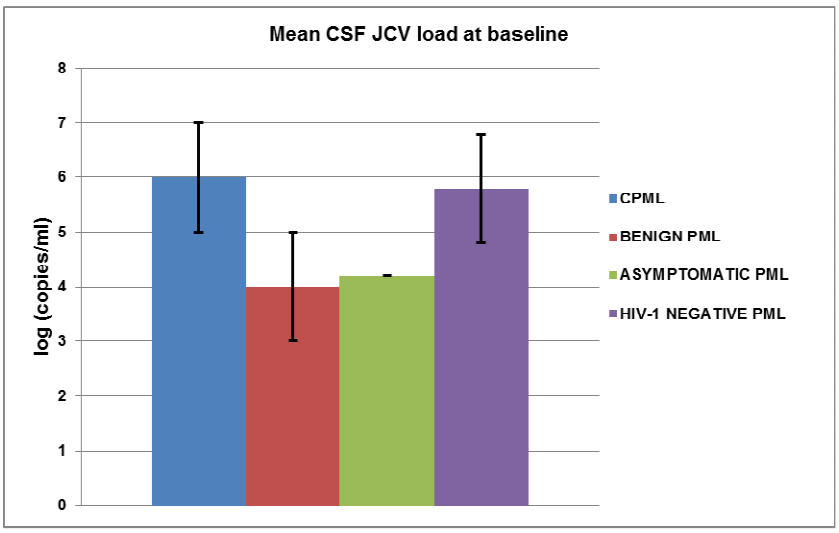


Fig. 20: mean JCV load in the CSF of PML patients at baseline.

4.1.3 Virological features of PML patients during follow-up

Follow-up was performed every six months, when possible, for virological analysis of collected biological samples. Follow up analysis was possible only for two CPML patients, six benign PML patients and all the three asymptomatic PML patients. In particular, the two CPML patients both had one follow-up examination six months after the enrolment, whereas the others CPML patients died within less than six months after PML diagnosis. The mean time of follow-up was 28 months (range: 6-60 months) for the six patients from the benign PML group and 16 months (range: 6-48 months) for the three patients in the asymptomatic PML group.
As can be observed in table 13, 14 and 15, and in fig. 21, 22 and 23, the JCV load in the CSF determined by means of Q-PCR has increased in three patients, in comparison to the baseline level, while decreased in each of the other patients. Four patients demonstrated virological remission, which is the clearance of the virus from CSF.
HIV-1 RNA was only detectable in the CSF samples from 3 PML patients during the follow-up, but all patients experienced the clearance of the virus during this period.
During the follow-up, JCV load was monitored also in PB and urine for benign and asymptomatic PML patients. In the PB, JCV was repeatedly detectable in one patients, and 3 follow-ups, it was detectable in one out of ten consecutive samples from another patient and was not detectable in the other patients. Regarding the JCV Q-PCR analysis in the urine, viral DNA was amplified in two of 11 follow-up examinations in one patients and was repeatedly detected in one of the asymptomatic PML patients (from 5 follow-up).

Table 13: CSF JCV load (log[copies/ml]) during the follow-up in CPML patients.

	Mean follow-up period: 6 months	
CPML	T0	T1
Patient#1	5.3	5.9
Patient#2	6.11	5

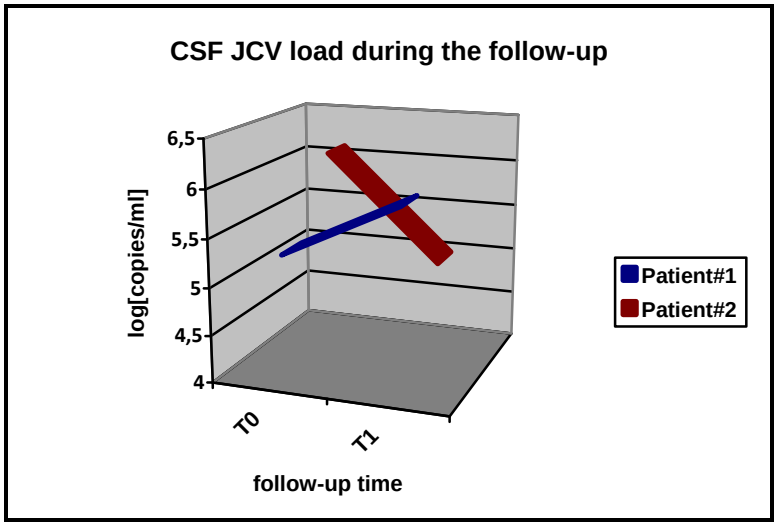


Fig. 21: CSF JCV load (log[copies/ml]) during the follow-up in CPML patients.

Table 14: CSF JCV load (log[copies/ml]) during the follow-up in benign PML patients.

	Mean follow-up period: 28 months (range: 6-60)						
Benign PML	T0	T1	T2	T3	T4	T5	T6
Patient#1	4.49	3.99	3.6				
Patient#2	6.32	5.27	3.23	3.23			
Patient#3	3.23	3.23	3.23	3.48	3.34	neg	neg
Patient#4	3.23	3.71	3.16	3.2	neg	neg	Neg
Patient#5	3.23	5.09	5				
Patient#6	4.05	5.7					

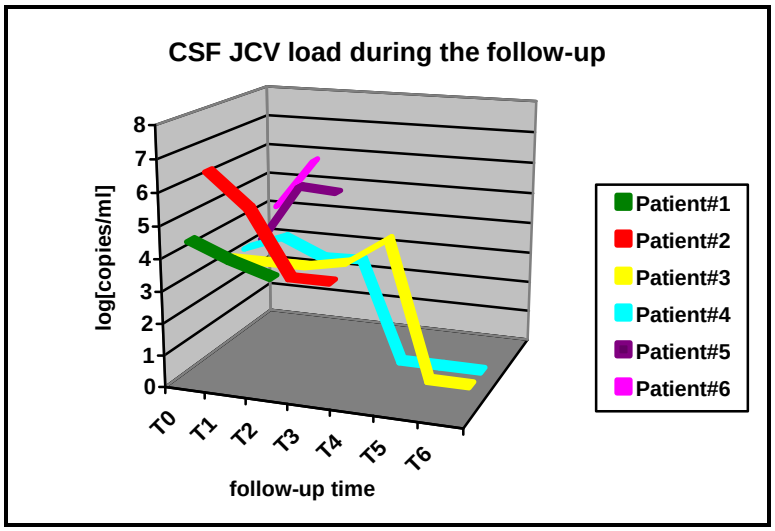


Fig. 22: CSF JCV load (log[copies/ml]) during the follow-up in benign PML patients.

Table 15: CSF JCV load (log[copies/ml]) during the follow-up in asymptomatic PML patients.

	Mean follow-up period: 16 months (range 6-48)				
Asymptomatic PML	T0	T1	T2	T3	T4
Patient#1	3.9	3.5	3.5	3.2	
Patient#2	4.8	5.4	3.2	3.2	neg
Patient#3	3.9	neg			

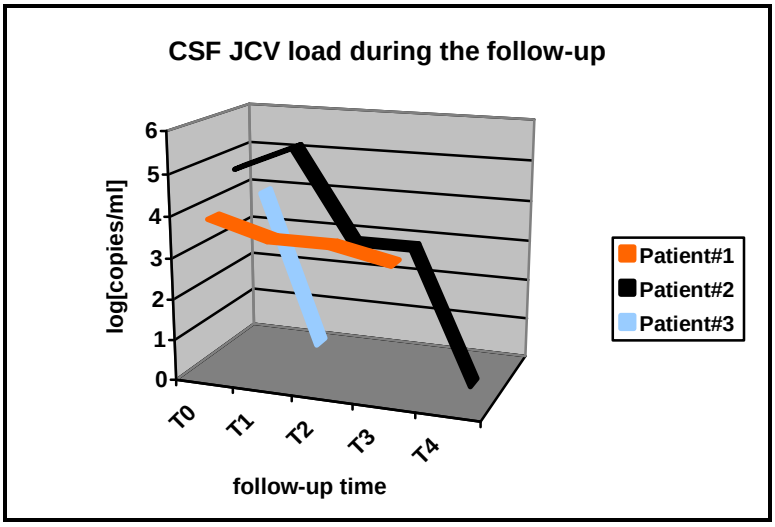


Fig. 23: CSF JCV load (log[copies/ml]) during the follow-up in asymptomatic PML patients.

Regarding the herpes viruses analysis on PML CSF samples during the follow-up period, no herpes virus genomes were amplified in PML patients.

4.1.4 Molecular characterization of JCV strains isolated from PML CSF

The molecular analysis of JCV strains amplificated by Q-PCR was performed only in CSF samples and the results are shown in table 16.
Eighteen CSF samples analyzed were identified as genotype 1, in particular 12 genotype 1a and 6 genotype 1b. Seven samples contained genotype 2, 4 genotype 2b and 3 genotype 2c. Finally, 1 sample contained genotype 4. There was not a significant difference in the distribution of these genotypes among PML patient group.

Table 16: results of the molecular characterization of JCV strains isolated from PML CSF.

		CPML	BENIGN PML	ASYMPTOMATIC PML	HIV-1 NEGATIVE PML
JCV GENOTYPE	**1a**	4	5	2	1
	1b	2	1	1	2
	2b	2	1	1	/
	2c	1	/	/	2
	4	/	1	/	1

The molecular analysis of TCR rearrangements was performed for 25 CSF samples at baseline. Twenty-three out of 25 have shown a rearranged TCR structure, in particular 4 IR rearrangements and 19 IIR rearrangements, while 2 samples from asymptomatic PML patients demonstrated an archetypal form, the IIS. The CPML patients and all but one of the HIV-1negative PML patients showed IIR rearrangements; one patient from the benign PMl group, one from the asymptomatic PML group and one from the HIV-1 negative PML group presented a Mad1 rearrangement, while one benign PML patient had a Mad4 rearrangement (Jensen and Major, 2001; Frisque et al., 1984).
The same results were obtained from the analysis of JCV strains isolated from CSF during the follow-up.

4.1.5 Demographic and virological features of NDLE patients

Twenty-four patients with HIV-1 related leukoencephalopathy (NDLE) were enrolled in this study, 16 male and 8 female. Ten patients were completely asytmptomatic, while the remaining 14 presented symptoms milder than in PML. For these patients, CSF virological analysis was performed in order to identify the presence of JCV or other neurotropic virus.
However, neither JCV nor human herpes virus genomes were amplified in the 24 CSF of NDLE patients.

4.2 Results II

4.2.1 Case study

From January 2010, a total number of 48 subjects from the Multiple Sclerosis Center, Mondino Hospital, Pavia were included in this longitudinal study. All these patients were affected from the relapsing form of MS and were treated with natalizumab therapy, due to the failure, for an inadequate response to or for not toleration, of the first line disease-modifying MS treatment.
Five out of 48 patients were lost during the follow-up period and thus excluded from the analysis of the results, because of reasons linked to natalizumab treatment, such as the onset of infectious complications and allergic reactions, or independent from the drug administration (as the end of the treatment).
PB, serum and urine samples were collected from each patient at various time-points during the treatment, in conjunction with monthly natalizumab infusion. Additionally, biological samples (PB, serum and urine) were collected once from an individual set of 25 MS individuals, enrolled as control group, affected from MS and not treated with natalizumab, but with a conventional immunosuppressive therapy, including Copaxone, Betaferon, Extavia, Avonex, Rebif or Azathioprine. These patients were treated from an average time of 2.5 years, ranging from 1 to 6 years.
Demographics, treatment duration, and description of the collected samples from natalizumab-treated and control MS patients are summarized in table 17.
The total number of natalizumab infusion for the natalizumab-treated patients was 1,254, with mean treatment duration of 27.95 months (range 3-59).
The biological samples were collected from natalizumab-treated patients for 741 time-points, with a mean time-point for each patient equal to 16.75 (range 2-29).
A total of 2,184 biological samples were collected from natalizumab-treated MS patients during the follow-up period, while 54 biological samples were collected from the reference group of control patients.
The 43 natalizumab-treated patients enrolled were classified into two distinct subgroups, on the basis of the treatment duration at the time of the analysis:

- Group A: 19 RR-MS patients, who underwent natalizumab infusion from 3 to 24 months (M/F: 5/14; mean age: 35, range: 18-52);
- Group B: 24 RR-MS patients, who underwent natalizumab infusion from 25 to 59 months (M/F: 8/16; mean age: 40, range: 25-55).

Demographics and treatment duration for Group A and Group B natalizumab-treated patients are summarized in table 18.
A total of 335 natalizumab monthly infusions have been performed for the patient of Group A, with a mean of 17.6 infusion (range 3-25) for each patient. The total number of time-points for Group A was 238, with an average of 12.5 (range 2-23).
For patients of Group B, a total of 919 monthly infusions have been carried out, with a mean of 38.3 infusion (range 25-59). The total number of time-points for Group B was 503 and the average 21 (range 8-29).

Table 17: demographics, treatment duration, and description of the collected samples from natalizumab-treated and control MS patients.

	Longitudinal Study	
	Natalizumab-treated patients	**Control group**
No.	43	28
Mean Age (range)	37.5 (18-55)	43.1 (21-66)
M/F	13/30	10/18
Mean treatment duration at analysis in months (range)	27.95 (3-59)	30 (12-72)
Mean time-point (range)	16.75 (2-29)	1
No. of samples	2,184 (766 PB, 761 serum, 657 urine)	54 (18 PB, 21 serum, 15 urine)

Table 18: demographics and treatment duration for Group A and Group B natalizumab-treated patients.

	Group A	**Group B**
Total patients	**43**	
No.	19	24
Mean Age (range)	35 (18-42)	40 (25-55)
M/F	5/14	8/16
Mean treatment duration at analysis in months (range)	17.6 (3-25)	38.3 (25-59)
Mean time-point (range)	12.5 (2-23)	21 (8-29)

4.2.2 Detection of JCV DNA in urine samples

A Q-PCR assay for JCV is used to detect viral DNA in urine samples from natalizumab-treated RR-MS and control patients.
Regarding the reference group, 7 out of 28 control patients (25%) are positive for JCV DNA in urine.
Among natalizumab-treated patients, 22 out of 43 patients (51.2%) had at least one positive PCR in urine and 215 time-points out of 743 were positive for the presence of JCV DNA.

Fisher test was used to obtain the exact p value and to define if results regarding the differences on urine JCV positivity in natalizumab-treated patients and in controls were statistically significant. The calculated p value was p=0.0472.
The mean JCV load in the urine of the control patients was log 6.2 copies/ml with a standard deviation of 1.5 (range: log 4.9- log 8.2 copies/ml), while the mean urine JCV load in natalizumab-treated group was log 7.2 copies/ml (standard deviation: 1.6; range: log 3.1- log 10.9) (table 19).

Table 19: results on the detection of JCV DNA in urine samples.

	Natalizumab-treated patients	**Control group**	**p**
JCV+ patients/ total	22/43	7/28	0.0472
JCV+ time-points/ total	215/743	-	-
Mean urine JCV load ± standard deviation (range)	log 7.2 ± 1.6 (log 3.1- log 10.9)	log 6.2 ± 1.5 (log 4.9- log 8.2)	0.1165

In table 20 are shown results on JCV positivity in urine analyzed for each of the natalizumab-treated group, Group A and Group B.
Regarding Group A, 9 patients out of 19 (47.4%) are at least once positive for the presence of JCV genome. JCV positive time-points are 67 out of 238 (28.15%). Group B positive patients were 13 out of 24 (54.2%), while 147 time-points out of 503 were positive (29.2%).
The mean JCV load for Group A was log 7.4 (range log 3.2- log 11) with a standard deviation of 1.7, and for Group B was log 7.2 (range log 3.15- log 7.8) with a standard deviation of 1.6.

Table 20: results on JCV positivity in urine analyzed for each of the natalizumab-treated group, Group A and Group B.

	Group A	Group B	p
JCV+ patients/ total	9/19	13/24	0.7626
JCV+ time-points/ total	67/238	147/503	0.7949
Mean urine JCV load ± standard deviation (range)	log 7.4 ± 1.7 (range log 3.2- log 11)	log 7.2 ± 1.6 (range log 3.15- log 7.8)	0.1653

4.2.3 Detection of JCV DNA in PB and serum samples

JCV was not detectable in any of the serum available specimens, neither at baseline nor during the treatment. One natalizumab-treated patient had a positive blood sample (1/766 total PB samples) at 21st natalizumab infusion, with a low viral load equal to 46.8 copies/µg. At the same time-point JCV DNA was detected also in the urine. This patient had always JCV positive samples during the treatment.
No PB samples from control patients were positive for JCV DNA.

4.2.4 Trend of JCV load in urine during the treatment

The total number of patients who were positive at baseline was 7/25 (28%). For further analysis, JCV positivity of RR-MS patients was classified during the first, second, third and fourth year of treatment (table 21 and fig. 24).
Two out of 42 natalizumab-treated RR-MS patients became JCV-DNA positive during natalizumab therapy either for a reactivation or for a new viral infection, while 1 patient demonstrated the clearance of the virus.
Six patients showed a sporadic reactivation of JCV in the urine, with viral detection in a maximum number of 3 time-points.
Regarding the trend of JCV load during the treatment for each patient, the viral load unvaried in 6 patients. For 5 patients, it increased its value of about one logarithm, whereas for as many patients it decreased of about one logarithm.

Table 21: JCV positivity of RR-MS patients classified during the first, second, third and fourth year of treatment.

Time of treatment	JCV+ Patients	Total patients	%
At baseline (T0)	7	25	28%
At 6 months (T6)	9	24	37.5%
At 9 months (T9)	9	24	37.5%
At 12 months (T12)	10	21	47.6%
At 18 months (T18)	11	27	40.1%
At 24 months (T24)	13	27	48.1%
At 30 months (T30)	7	22	31.8%
At 36 months (T36)	4	14	28.6%
At 42 months (T42)	3	15	20%
At 45 months (T45)	2	10	20%

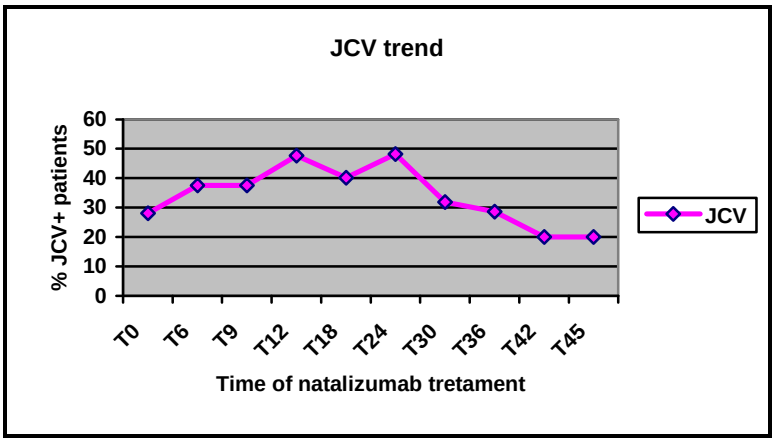
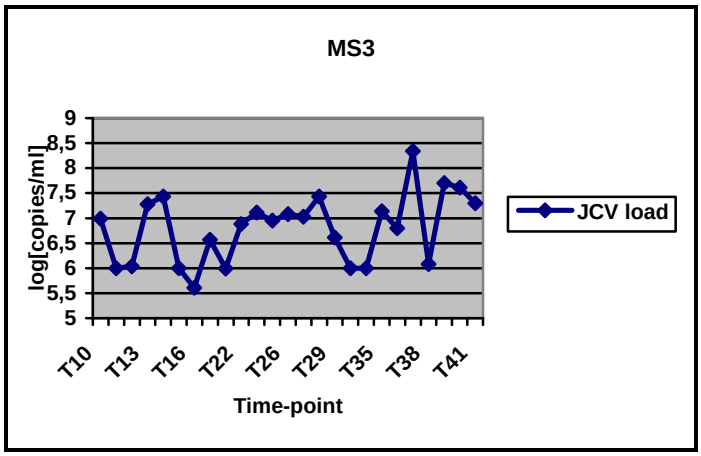
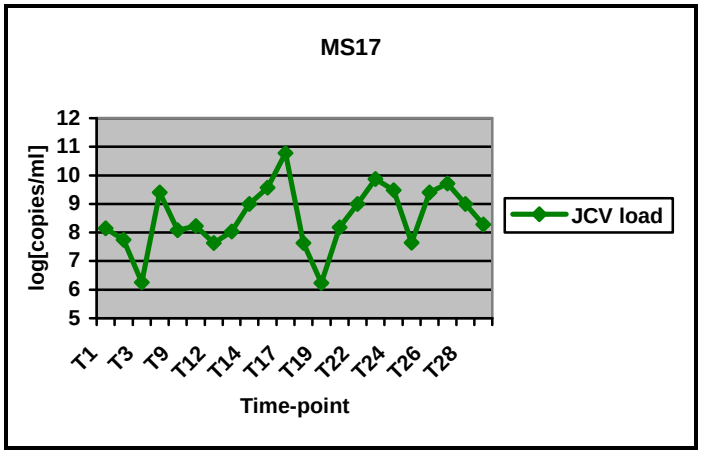
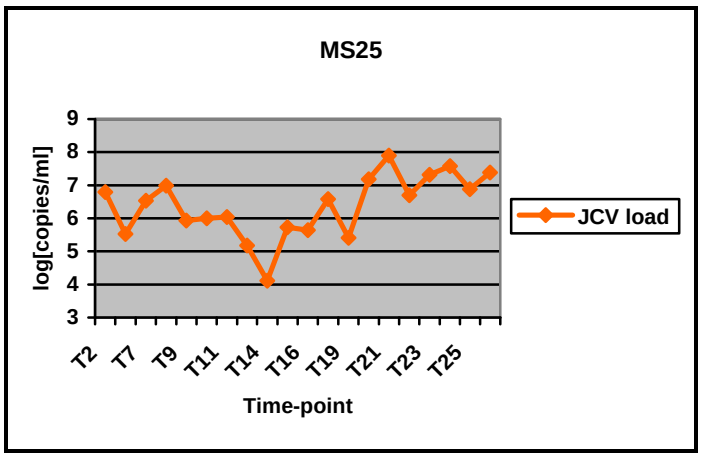


Fig. 24: trend of JCV load in urine during the treatment.

The trend of JCV load for each patient during the follow-up is represented in the following graphics.
Representation of the unvaried JCV load for 6 patients:



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T2
T7
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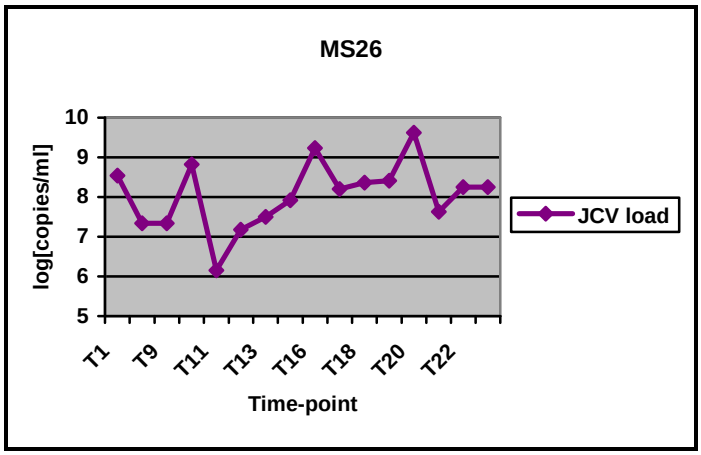
T11
T14
T16
T19
T21
T23
T25
Time-point
JCV load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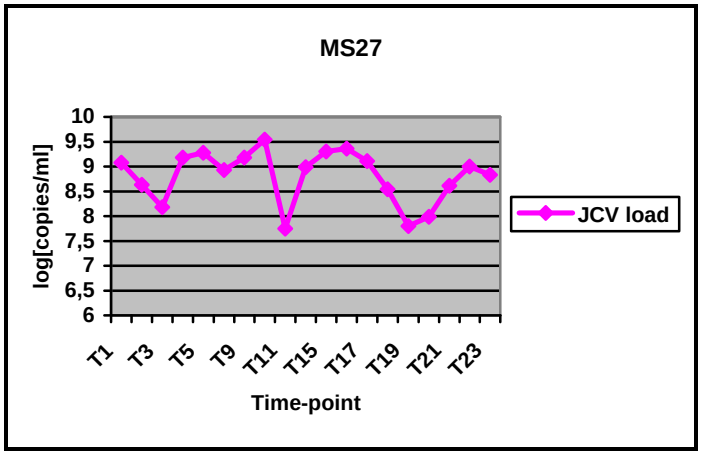
Time-point
JCV load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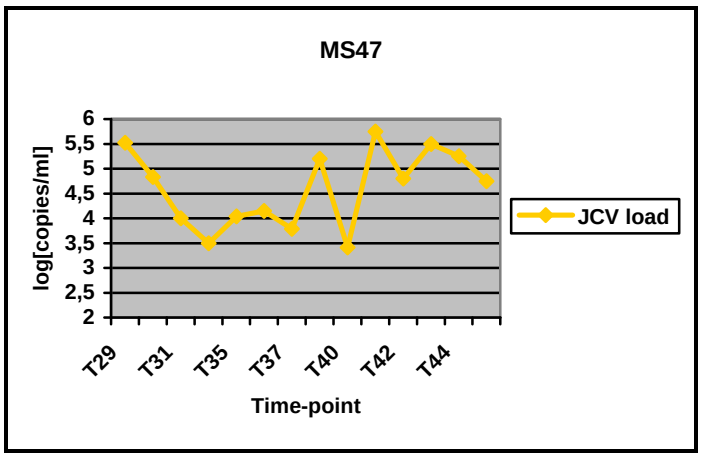
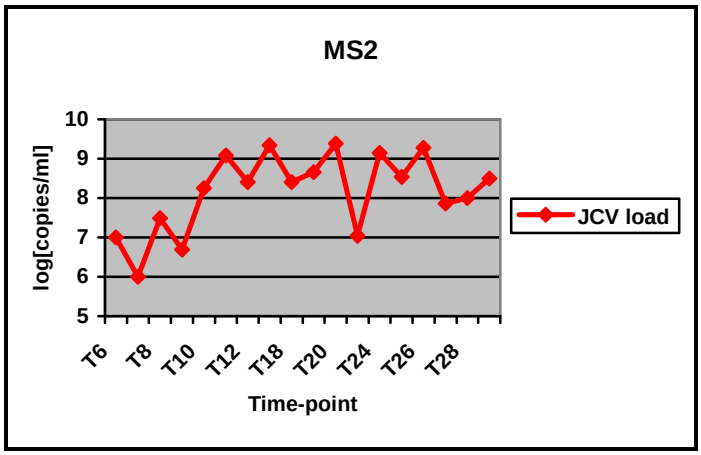
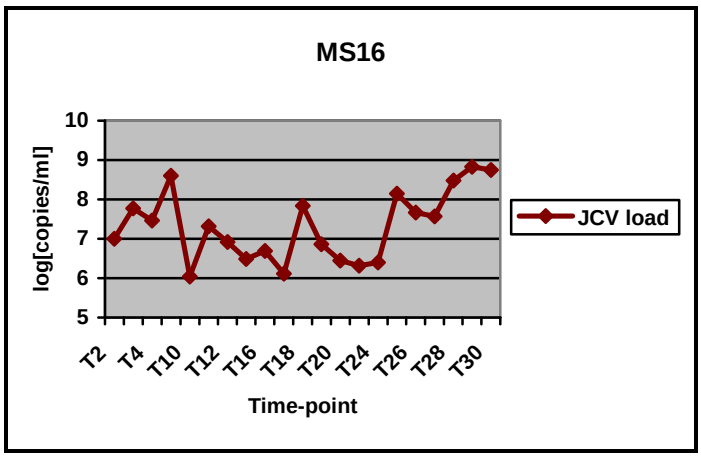
T15
T17
T19
T21
T23
Time-point
JCV load



Representation of the increased JCV load of about one logarithm:

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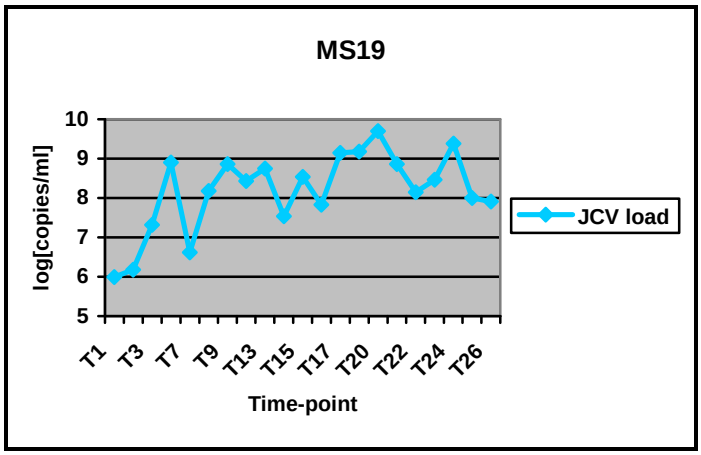
T26
T28
T30
Time-point
JCV load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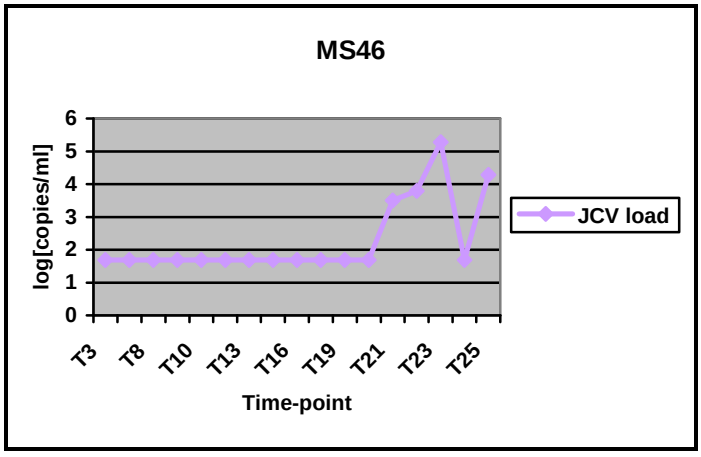
T24
T26
Time-point
JCV load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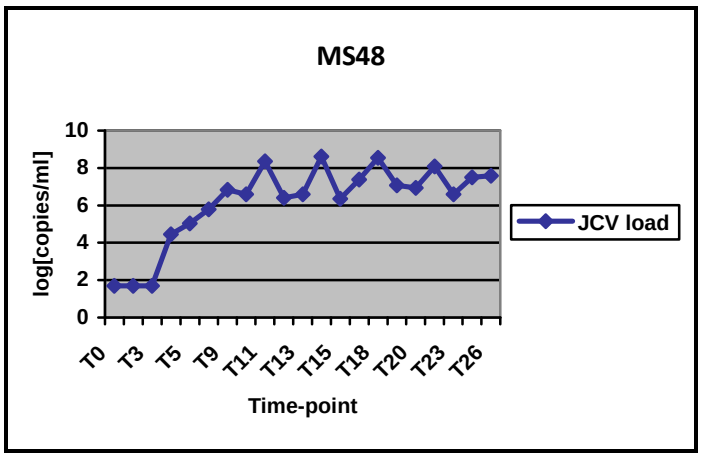
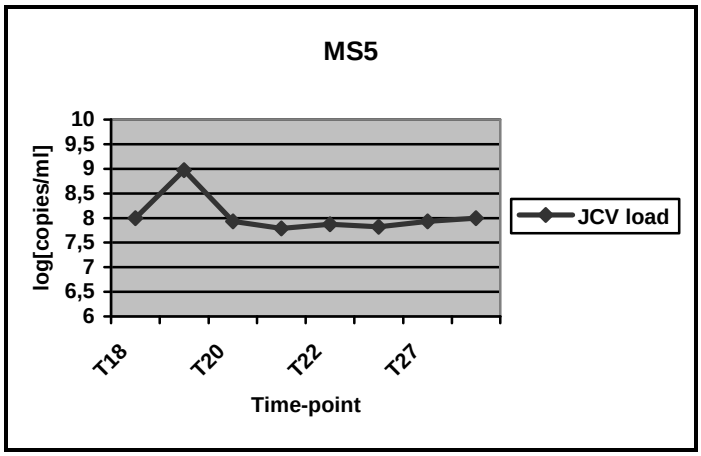
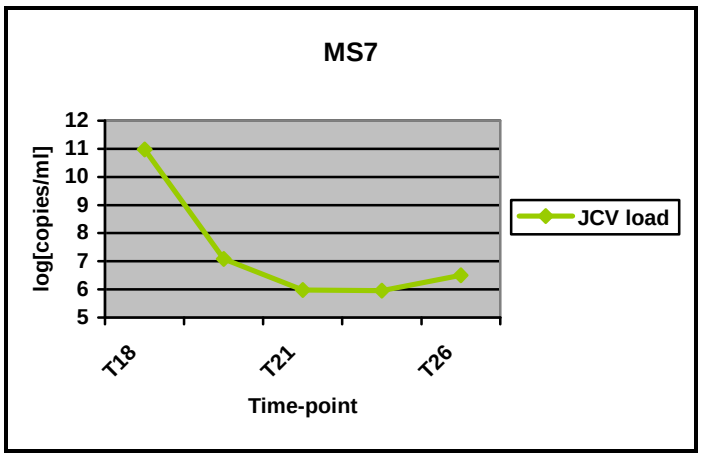
T25
Time-point
JCV load



Representation of the decreased JCV load of about one logarithm:

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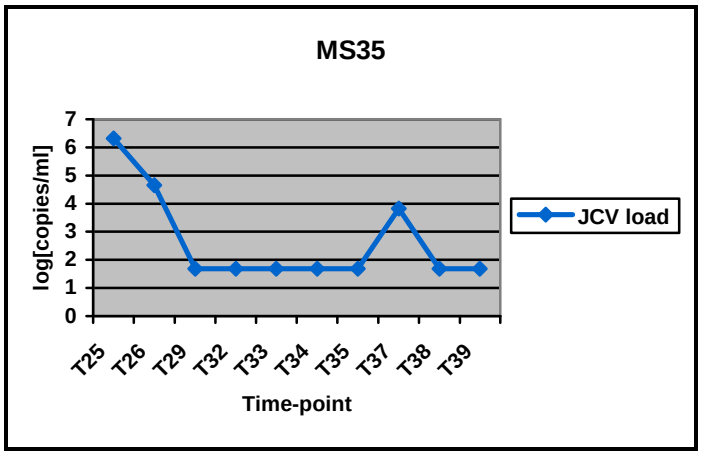
T26
Time-point
JCV load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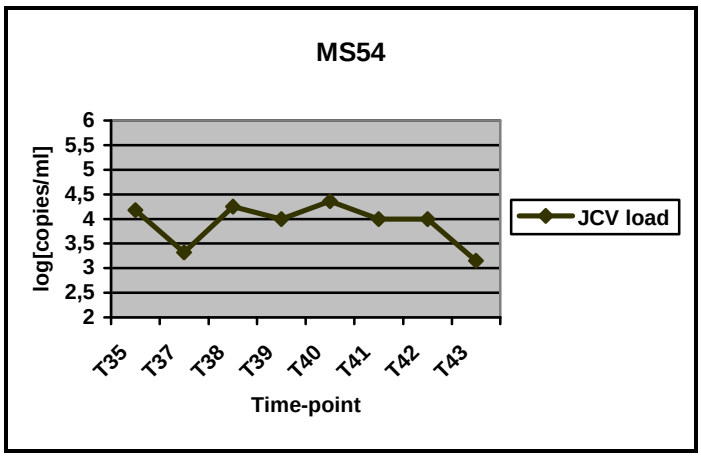
T39
Time-point
JCV load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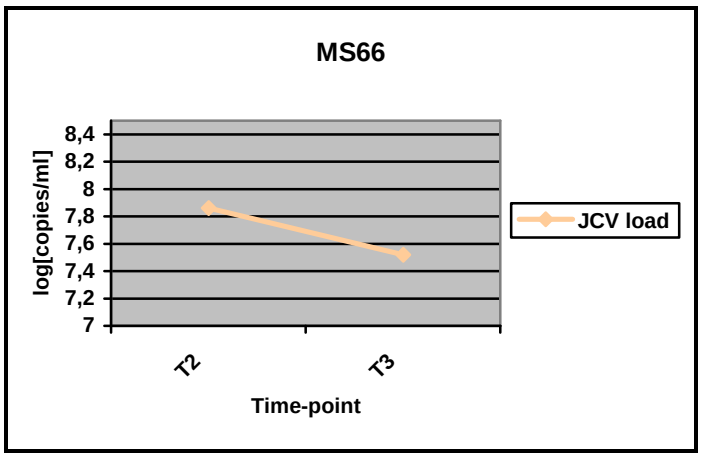
Time-point
JCV load



4.2.5 Molecular characterization of JCV strains isolated from urine samples

The genotyping analyses of the VP1 region have shown a predominance of the genotype 1, detected in 15 JCV positive urine samples of natalizumab-treated RR-MS patients. In particular, 9 urine samples were identified as genotype 1a and 7 as genotype 1b. Genotype 4 was identified in 3 JCV-positive urine samples; genotype 2b and 2c were amplified in two samples each (table 22).
Regarding the genotyping results of the control group, two urine samples contained genotype 1b, and two other samples were identified as genotype 1a and 4, respectively (table 22).
JCV TCR organization was analyzed both in natalizumab-treated patients and in control patients, showing an archetypal form in all the considered samples (table 22).

Table 22: results of the molecular characterization of JCV strains isolated from MS urine samples.

JCV VP1	Natalizumab-treated patients (Tot: 22 JCV+)	Control Group (Tot: 7 JCV+)
Gen. 1a	9	1
Gen. 1b	5	2
Gen. 2b	2	0
Gen. 2c	2	0
Gen. 4	3	1
JCV TCR	**Natalizumab-treated patients (Tot: 22 JCV+)**	**Control Group (Tot: 7 JCV+)**
Archetype	22	5

4.2.6 Detection of herpes virus genomes in PB and serum samples

The presence of herpes viruses EBV, VZV, HSV-1 and HHV-6 has been monitored in serum samples from natalizumab-treated patients and control patients. None of these viruses has ever been amplified in the available specimens.
EBV genome has been searched also in PB samples by means of Q-PCR both at baseline and during the follow-up period, and it has been detected sporadically, with a percentage of EBV-positive PB samples equal to 22.2%.

4.2.7 Serological reactivity to JCV

The serological test STRATIFY has been performed for 38 out of 43 natalizumab-treated RR-MS patients. Seventeen patients out of 38 (44.7%) have detectable anti-JCV antibodies in serum, whereas the remaining (55.2%) resulted negative at anti-JCV antibodies test. The latters underwent a re-test, one year from the first STRATIFY, and 12/16 patients already subjeted to the second test resulted negative at both test. 4/16 patients showed an anti-JCV antibodies positivity at the second ELISA test, while 5 out of 21 resulted negative at first STRATIFY must yet be tested for the second time. Regarding the twelve seronegative patients, 2/12 has already been tested for the third time and has showed a negative result.

The results on the correlation between urinary JCV-DNA and anti-JCV antibodies status determined at the first STRATIFY are showed in the table 23.

Table 23: correlation between urinary JCV-DNA and anti-JCV antibodies status determined at the first STRATIFY test.

	Urinary JCV-DNA (+) (22)	**Urinary JCV-DNA (-) (21)**
Anti-JCV antibodies (+) (17)	15	2
Anti-JCV antibodies (-) (21)	3	18

As shown in the table, 15 out of 38 patients resulted postive both at urinary JCV DNA test and at anti-JCV antibodies test. Eighteen out of 38 resulted negative at both test. Three out of 22 natalizumab-treated patients with detectable JCV DNA in the urine resulted negative at anti-JCV antibodies test. This result represents a false-negative of the STRATIFY test, and it could demonstre the lower sensibility of ELISA test compared to Q-PCR. Finally, two patients showed positivity to anti-JCV antibodies in the absence of JCV DNA in the urine.

5. Discussion

Here a wide molecular and longitudinal study is presented aimed to give a significant contribution to the understanding of the molecular basis of demyelinating diseases pathogenesis.

MS is a multifocal demyelinating disease with progressive neurodegeneration caused by an autoimmune response to self-antigens in genetically susceptible individuals. Patients often exhibit an initial clinically isolated syndrome, followed by a series of sub acute clinical events that spontaneously abate, referred to as a RR form of the pathology. At present, no cure exists for MS and the goals of treatment are to arresting or slowing the progression of disability, decreasing relapse rate, managing symptoms, and slowing subclinical disease progression. The most commonly used MS treatments are immunomodulating agents, such as IFNβ-1a, IFNβ-1b, GA and fingolimod. mAbs, which can target pathogenic pathways with higher specificity, higher potency and fewer adverse effects than other molecules, may have great potential as therapies for autoimmune diseases. Their development as treatments for MS is promising, mainly for patients failing classical immunomodulatory treatments, representing an important new treatment option. In particular, Natalizumab (Tysabri; Biogen-Idec, Elan Pharmaceuticals, USA), a humanized mAb directed against the α4 subunit of the adhesion molecule α4β1 integrin, is the only mAb approved for the treatment of MS. Natalizumab reduces leukocytes ability to migrate across the BBB, and based on the results of two phase III clinical trials it was approved on November, 2004 for the treatment of highly active or rapidly evolving severe RR-MS. On February 2005, natalizumab was withdrawn voluntarily from the market after the occurrence of Progressive Multifocal Leukoencephalopathy (PML) in three patients, treated with the mAb in clinical trials for MS and Crohn's disease, representing an incidence of approximately 1 case per 1,000 patients. Following the implementation of a risk management strategy, the US FDA and the EMA remarketed natalizumab as monotherapy for MS in July 2006, with recommendations for monitoring for new cases of PML.

PML is an opportunistic demyelinating disease caused by JCV, most often in the context of immunodeficiency. PML remains an incurable and often fatal disease, although the wide use of antiretroviral treatment led to a significant reduction of mortality in HIV-1 positive PML patients from 90% to approximately 50% during the first three months of treatment, as a result of recovery of the immune system. However, the introduction of HAART was associated to the development of a PML-like leukoencephalopathy, indicated as NDLE. This pathology is characterized by the presence of demyelinating lesions and mild symptoms resembling those of PML but in the absence of the JCV genome in the CSF.

As of October 3, 2012 there have been 298 confirmed cases of PML among 108,300 natalizumab-treated MS patients, with 63 deaths and a mortality rate of 21.1%. Additionally, accumulated safety data from global risk management program, established at the time of natalizumab reintroduction on the market, have identified longer duration of natalizumab treatment, the use of immunosuppressants before the initiation of natalizumab therapy, and positive status with respect to anti–JCV antibodies, as assessed with the use of a two-step anti-JCV antibody assay (STRATIFY JCV, Focus Diagnostics), as risk factors for

PML [235,239,251]. The risk of PML was highest among MS patients who had all these three risk factors (positivity to anti-JCV antibodies, prior use of immunosuppressants, and natalizumab treatment for 25 to 48 months), with an estimated incidence of 11.1 cases per 1,000 patients.

Although there are several differences between natalizumab-associated PML compared with PML in the contexts of immunodeficiency or malignancy, the common feature of demyelination has stimulated the research on the possible association between MS, JCV reactivation and PML. It is evident that there is still a need to identify markers for PML prognosis and diagnosis in natalizumab-treated MS patients, and a need for the definition of a screening test which can reliably and easily predict PML.

To understand the mechanism of reactivation of a latent infection in natalizumab-treated patients, its therapeutic principles of action must be considered. Natalizumab was specifically designed to reduce trafficking of lymphocytes into peripheral tissues; therefore, it was postulated that treatment with natalizumab results in reduced immune surveillance of the CNS. Two possible mechanisms of JCV reactivation are reported in the literature. Either the persisting virus within the CNS or passing virus during JC viremia is responsible for JCV reactivation in the setting of immunosuppression or impaired immunosurveillance [reviewed in 108]. In addition, since natalizumab forces hematopoietic stem cells and pre-B-cells to migrate from the bone marrow, because of they cannot bind to vascular-adhesion molecules, patients with MS who receive natalizumab treatment have an increase in CD34+ cells in their circulation as well as an up-regulation of genes involved in B-cell maturation [252-254]. This dynamic creates a favorable environment for JCV, which can reside in a latent state in the bone marrow for long periods before the development of PML and which can use B-cells and their DNA-binding proteins to initiate viral replication. In fact, natalizumab up-regulates transcription factors important for the differentiation of B lymphocytes and, during natalizumab-induced B-cell differentiation, JCV-infected bone marrow cells might be activated, leading to JC viremia and PML as a consequence of natalizumab therapy. In this context, rearrangement of archetype JCV to PML-type could occur [255-257].

Since the presence of JCV DNA in body fluids is thought to represent active viral shedding or replication and since blood testing and urine analysis are less invasive than CSF testing, they are an attractive option to reveal some clues about JCV reactivation in MS patients. Previously published studies have reported conflicting information on the effect of natalizumab on JCV DNA presence in biological fluids [230,242,244,245].

Thus, to better understand the molecular basis of demyelination, and to identify possible risk factors for the development of opportunistic infections and early prognostic markers of infectious complication during natalizumab treatment of MS, a wide longitudinal study has been performed, based on the collection of biological fluids from MS patients subjected to natalizumab or other treatment, and from PML and NDLE patients. CSF, PB, urine and serum samples have been collected at various time-points and JCV genome searched, quantified and molecular characterized in order to monitor the viral presence and load, and to determine wheter they could be considered as risk factors for PML development.

Fifty patients with brain lesions and clinical symptoms suggestive of PML and NDLE have been enrolled in this study. CSF, urine and PB samples from these patients have been virologically evaluated, in order to monitor the presence of JCV and other neurotropic viruses both at baseline and during the follow-up.
In particular, 22 HIV-1 positive PML patients were classified into three different groups on the basis of the symptomatology at the onset and during the progression of the disease at follow-up: 10 patients were included in the classical PML group, 9 patients in the benign PML group and 3 patients in the asymptomatic PML group. Six HIV-1 negative PML patients, affected by onco-hematological malignancies, were also analyzed. Follow-up analysis were possible for only two CPML patients (mean follow-up period: 6 months), since eight patients died within few months, for six benign and the three asymptomatic patients (range of follow-up: 6-60 months), and for any of the HIV-1 negative PML patients. The analysis of immunological and virological features at baseline demonstrated that CPML patients had the lowest CD4+ cells count followed by the benign and the asymptomatic PML patients. The analysis of virological features performed at baseline by means of Q-PCR has shown that the mean JCV load in the CSF was significantly higher in the CPML group than in the other HIV-1 positive PML groups, and was very high also in the group of HIV-1 negative PML patients. These virological findings seem to suggest that factors related to both the JCV infection (high CSF viral load) and the host immune system (low CD4+ cell count) can influence the prognosis of the disease [reviewed in 250]. Additionally, they support previously published data, which reported a poor prognosis for HIV-1 negative PML patients. Indeed, of the HIV-1 negative PML patients, three died within few months from disease onset, and two other are still alive, but with severe neurological conditions.
JCV analysis during the follow-up has indicated that JCV load remained stable in two CPML patients, was decreased in four of the six benign PML patients and was decreased in all of the asymptomatic PML patients. The clearance of the virus from the CSF was evident in four cases. These data support previous findings reporting that the viral clearance occurs after 5 to 25 months of HAART and that a lowering of JCV replication in the brain is related to prolonged survival rate [258-259].
The slow progression of the disease that characterized the group of benign and asymptomatic PML patients was confirmed also by the clearance of HIV-1 RNA from the CSF.
The viral strain amplified in CSF were molecular characterized by standard PCR, but no mutation or large change occurred in the viral genome organization neither at baseline nor during the follow-up. The genotyping analysis of VP1 region have shown a prevalence of genotype 1, confirming the geographical distribution of the viral strain, while the analysis of TCR rearrangements have shown a large majority of IR and IIR types, as expected. Nineteen of the IIR TCR were identified as different from all the other TCRs regarding sequence and length, and as unique in the NCBI database. Three JCV TCR sequences were found as Mad-1, the PML-type strain, and one as Mad-4. The most interesting result, comes from the observation that the archetypal form IIS, usually isolated from urine of healthy and PML subjects and without an apparent infectious activity in vitro, was amplified from the CSF of two asymptomatic PML patients. The potential role of the different

molecular organizations of the JCV TCR in PML pathogenesis and in viral neurotropism has been long debated, and, to date, the rearranged forms of TCR seem to play a crucial role in PML pathogenesis [reviewed in 250].
On the basis of this finding, it is possible to assert tha the presence of a weaker promoter, such as the archetype, and the consequently low viral replication in the CNS of asymptomatic PML patients may partially explain the benign course of the disease.
The detection of JCV DNA at baseline in PB and urine samples from PML patients did not show statistically significant results, and this is also for the analysis during the follow-up period.
No herpes virus genomes were amplified in the CSF of PML patients, so these viruses seem not to be involved in PML pathogenesis.
Twenty two HIV-1 positive patients affected by NDLE were enrolled and followed-up, and it was confirmed that none of the searched neurotropic viruses was involved in the pathogenesis of this form of leukoencephalopathy, since neither JCV nor herpes viruses were found in the CSF from these patients.
In order to investigate the role of JCV and other neurotropic viruses in PML onset during the natalizumab therapy in MS patients, seventy-six MS patients were recruited and PB, serum and urine were collected at the enrolment and monthly during the follow up to evaluate the viral presence. Forty-eight of these were affected by the RR form of the pathology and treated with the mAb natalizumab, while the others were enrolled as control group and have been treated from at least two years with the conventional disease-modifying therapy, such as INFβ, GA or azathioprine.
Five of 48 RR-MS patients were dropped out from natalizumab therapy and from the analysis, because of the onset of complications not compatible with mAb treatment. The remaining 43 natalizumab-treated RR-MS patients were classified in two subgroups, in order to verify JCV replication within 24 months of therapy administration (time at which the PML risk is higher) and with the treatment continuation.
No enrolled patients developed clinical or brain MRI evidence typical of PML.
Q-PCR assay was performed to investigate JCV presence and viral load in PB, urine and sera collected at baseline and during the monthly time-points, concomitant to monthly infusion of the drug.
Viral replication has been often detected in urine samples, while the viral genome was amplified only in one PB sample and no serum samples resulted positive for JCV. Comparing the findings in the case and the control groups, it seems that the virus replicates more in natalizumab-treated patients than in controls, but the urinary viral load remains comparable between the two groups.
Regarding the comparison between the rate of JCV positivity among natalizumab-treated patients and the time of treatment (number of monthly infusion), it appears that there is an increase of positivity until the 24th infusion/ month of treatment, and a decrease from the 36th infusion. This is in agreement with the findings of other reports, which describe a maximum risk for PML development during the first two years of natalizumab therapy [235,260,261]. A report by Chen et al. [230] suggests that the virus is shed in the urine in increasing amounts between 6 and 12 months

after natalizumab treatment begins, and this process may lead to viremia following treatment for 18 months. Sadiq et al. [245] reported that 3 of 200 patients had detectable JCV DNA. The JCV DNA detection in plasma occurred at the 6-month analysis, at 4 months of treatment for the second case, and the other JCV DNA case occurred after 18 months of natalizumab treatment. Jilek et al. did not show increased occurrence of JCV DNA in plasma, PBMCs, or urine in 24 patients treated with natalizuab for 18 months [244]. Rudick and colleagues analysed plasma, urine and PBMCs at baseline and after 48 weeks of natalizumab treatment, and their results did not confirm an induction of JCV DNA in body fluids [242].

It is important to underline that nobody have investigated the prevalence of urinary JCV DNA in urine of patients treated for more than 18 months, while this may be of great relevance, since most of PML cases occur after 2 years of treatment.

All patients had undetectable herpes virus DNA both at baseline and at follow-up in the serum, while EBV genome was amplified occasionally in PB samples. Thus far, there are only a few reports on the reactivation of neurotropic latent virus other than JCV in natalizumab-treated patients. HHV-6 is a pleiotropic β-herpes virus commonly reactivated in the setting of acute and prolonged immunosuppression. HHV-6 has also been suggested to be associated with PML pathogenesis, and to be involved in the pathogenesis of MS, as well as EBV [reviewed in 108]. Nevertheless, on the basis of the results of this study, herpes viruses seem not to reactivate during natalizumab treatment.

A comparison between the prevalence of urinary JCV DNA in natalizumab-treated patients and the serostatus with respect to anti-JCV antibodies has been performed, in order to evaluate the utility in testing JCV DNA togheter with anti-JCV antibodies in these patients as a tool for PML risk stratification.

All MS patients subjected to natalizumab therapy compulsorily undergo the two-step anti-JCV antibody assay STRATIFY, because seropositivity for anti-JCV antibodies, togheter with treatment duration >2 years and previous therapy with immunosuppressants, has been associated with an increased risk for PML development. This test is characterized by a false negative rate of 2.5%. In addition to obvious explanations for the false-negative results, such as sample mishandling, there are several biological explanations. First, the level of antibodies in the sample may be below the level of detection due to a very recent infection with the virus. Second, a low viral level may be present in the tested patients. Third, a false-negative response may be due to the patient’s antibodies being directed to epitopes not represented in the MAD-1 JCV VLPs used in the assay. The most plausible explanation is an intrinsic variance among individuals in antibody response to JCV.

Moreover, the false-negative rate, combined with the annual seroconversion rate (estimated as ~2% in the STRATA study) suggests that periodic retesting of seronegative patients is warranted in the clinical practice.

This ELISA test has been performed for 38/43 natalizumab-treated MS patients enrolled in this study. 17/38 (44.7%) patients had JCV antibodies. The patients resulted regative at anti-JCV antibodies test were re-tested one year after, and 16/21 patients already re-tested had not anti-JCV antibodies. Interestingly, a

seroconversion was shown for 4/16 (25%) patients, because they resulted positive, on the contrary to the first STRATIFY. This result may be due to a new infection with JCV in patients previously negative or, alternatively, could be proposed an explanation related to the test sensitivity. In fact, from March, 2012 the two-step ELISA assay developed by Gorelik and colleagues [239] characterized by a false-negative rate of 2.5% has been replaced with an improved STRATIFY JCV assay, called STRATIFY DxSelect. This second generation test has an improved ability to detect seropositivity in samples with low levels of anti-JCV antibodies so that it should minimise the occurrence of false negative results. Biogen Idec anticipated that approximately 5-10% of MS patients with very low anti-JCV antibody levels who tested negative using first generation STRATIFY JCV test may test positive with STRATIFY DxSelect. As a result, there may be an increase in the number of MS patients who had previously tested anti-JCV antibody negative prior to March 2012 and now test anti-JCV antibody positive at a rate above what might be expected from the scientific literature. Thus, it is expected that once MS patients have been tested with STRATIFY JCV DxSelect, the rate of changing serostatus stabilize.

For the natalizumab-treated MS patients of this study, the anti-JCV antibodies evaluation has been performed with the first generation assay until March, 2012. Thus, the most recent test has been characterized by more sensitivity, which can explain the apparent seroconverion. So, it can be postulated that these 4 patients were JCV positive also at the time of the first test, but its lower sensitivity may have influenced the result, or that the seroconversion really happened.

This result has a great clinical relevance, because patients with anti-JCV antibodies may have an increased risk to developed PML, in the presence of the other risk factors. It is fundamental that patients with negative anti-JCV antibodies are re-tested, at periodic intervals.

Regarding the twelve patients seronegative also at the second test, 2/12 has already been tested for the third time and shown again negative results.

Regarding the correlation between the urinary JCV DNA and anti-JCV antibodies, 15 out of 38 (39.5%) tested patients were postive both at urinary JCV DNA test and at anti-JCV antibodies test. 18/38 (47.4%) resulted negative at both test. Three out of 22 natalizumab-treated patients with detectable JCV DNA in the urine resulted negative at anti-JCV antibodies test, both at the first test and at the re-test. This result represents a false-negative of the STRATIFY test, and it may be explained by the lower sensitivity of the ELISA test compared to the Q-PCR test, or alternatively by an inter-individual difference relative to the immune response to JCV infection. In these analyses, the anti-JCV antibodies test showed a false-negative rate of 7.9% but an higher sensibility (44.7% positive patients) in comparison to urinary JCV DNA (40.8%). Finally, two patients showed positivity to anti-JCV antibodies in the absence of JCV DNA in the urine, probably due to an intermittent excretion of JCV DNA. In fact, when JCV establishes latency in the body, it is not shedded in the urine except in the case of reactivation or active viral replication.

In conclusion, on the basis of the results of this longitudinal study on different demyelinating diseases it is possible to assert that, to date, the main markers of

prognosis of HIV-related PML seem to be the JCV load in the CSF determined at the time of the disease, a low CD4+ cell count at baseline (associated with a severe clinical course) and the TCR molecular organization, showing anomalous structure in the CSF of long-term survival patients. In addition, the clearance of JCV from the brain, also observed in long-term survival patients, is probably due to an improved immunological status. The same asserts can be sustained for PML associated to hematological malignancies, since the JCV load and the TCR molecular organization in HIV-1 negative patients correlated with the poor prognosis.

This observation is alarming, because of the development of PML in HIV-1 negative subjects is often related to the administration of new drugs, based on mAbs. Although JCV reactivation detected in urine from MS patients had a subclinical nature, the increase of viral replication during natalizumab treatment cannot be ignored and the constant monitoring of urinary JCV DNA allows identifying patients who harbor the virus and verifying if viral replication/shedding in the urine is really influenced by the treatment with the drug. Thus, testing urinary JCV DNA, together with anti-JCV antibodies, could be of particular utility for indentifying individuals at risk of PML, also among patients who resulted negative at anti-JCV antibodies test. The experience with natalizumab argues that directed investigation aimed at identifying biomarkers associated with an elevating risk for PML has a great importance, becoming the major challenge of the research in the near future.

6. References

1. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000 Sep 28;343(13):938-52.
2. http:// mssociety.org.uk/what-is-ms/types-of-ms/relapsing-remitting-rrms, accessed on 25/10/2012.
3. http:// msqld.org/about-ms/types-of-ms, accessed, accessed on 25/10/2012.
4. Comabella M, Khoury SJ. Immunopathogenesis of multiple sclerosis. Clin Immunol. 2012 Jan;142(1):2-8. doi: 10.1016/j.clim.2011.03.004.
5. Demyelinating diseases. In: Clinical Neuropathology: text and color atlas. Author: Catherine Haberland. Pub: Demos Medical. Pp: 158-168, Ch.8
6. Disanto G, Morahan JM, Barnett HM, Giovannoni G, Ramagopalan SV. The evidence for a role of B-cells in multiple sclerosis. Neurology. 2012 Mar 13;78(11):823-32.
7. Weinshenker BG, Rodriguez M. Epidemiology of multiple sclerosis. In: Gorelick PB, Alter M, eds. Handbook of neuroepidemiology. Vol. 29 of Neurological disease and therapy. New York: Marcel Dekker, 1994:533-67.
8. Libbey JE, Fujinami RS. Potential triggers of MS. Results Probl Cell Differ. 2010;51:21-42.
9. http://biogenidec.it/epidemiologia_della_sm.aspx?ID=9775, accessed on 26/10/12
10. Boppana S, Huang H, Ito K, Dhib-Jalbut S. Immunologic aspects of multiple sclerosis. Mt Sinai J Med. 2011 Mar-Apr;78(2):207-20. doi: 10.1002/msj.20249.
11. Oksenberg JR, Baranzini SE. Multiple sclerosis genetics--is the glass half full, or half empty? Nat Rev Neurol. 2010 Aug;6(8):429-37. Epub 2010 Jul 13.
12. Hillert J, Olerup O. HLA and MS. Neurology. 1993 Nov;43(11):2426-7.
13. International Multiple Sclerosis Genetics Consortium, Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, de Bakker PI, Gabriel SB, Mirel DB, Ivinson AJ, Pericak-Vance MA, Gregory SG, Rioux JD, McCauley JL, Haines JL, Barcellos LF, Cree B, Oksenberg JR, Hauser SL. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med. 2007 Aug 30;357(9):851-62.
14. Kurtzke JF. Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev. 1993 Oct;6(4):382-427.
15. Kurtzke JF, Heltberg A. Multiple sclerosis in the Faroe Islands: an epitome. J Clin Epidemiol. 2001 Jan;54(1):1-22.
16. Correale J, Ysrraelit MC, Gaitán MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. Brain. 2009 May;132(Pt 5):1146-60.
17. Sobel R, Moore GRW. Demyelinating diseases. In: Love S, Louis DN, Ellison DW, eds. Greenfield's Neuropathology. 2008;vol. 2, pp. 1513-1594. New York: Oxford University Press.
18. Lassmann H, van Horssen J. The molecular basis of neurodegeneration in multiple sclerosis. FEBS Lett. 2011 Dec 1;585(23):3715-23. Epub 2011 Aug 16.
19. Jack C, Ruffini F, Bar-Or A, Antel JP. Microglia and multiple sclerosis. J Neurosci Res. 2005 Aug 1;81(3):363-73.

20. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, Laursen H, Sorensen PS, Lassmann H. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain. 2009 May;132(Pt 5):1175-89.
21. Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol. 2000 Jun;47(6):707-17.
22. Brück W. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. J Neurol. 2005 Nov;252 Suppl 5:v3-9.
23. Hu W, Lucchinetti CF. The pathological spectrum of CNS inflammatory demyelinating diseases. Semin Immunopathol. 2009 Nov;31(4):439-53.
24. Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, Olsson T, Linington C, Schmidbauer M, Lassmann H. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am J Pathol. 2000 Jul;157(1):267-76.
25. Popescu BF, Lucchinetti CF. Pathology of demyelinating diseases. Annu Rev Pathol. 2012;7:185-217.
26. Stadelmann C, Kerschensteiner M, Misgeld T, Brück W, Hohlfeld R, Lassmann H. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? Brain. 2002 Jan;125(Pt 1):75-85.
27. Barkhof F, Bruck W, De Groot CJ, Bergers E, Hulshof S, Geurts J, Polman CH, van der Valk P. Remyelinated lesions in multiple sclerosis: magnetic resonance image appearance. Arch Neurol. 2003 Aug;60(8):1073-81.
28. Patrikios P, Stadelmann C, Kutzelnigg A, Rauschka H, Schmidbauer M, Laursen H, Sorensen PS, Brück W, Lucchinetti C, Lassmann H. Remyelination is extensive in a subset of multiple sclerosis patients. Brain. 2006 Dec;129(Pt 12):3165-72.
29. Brück W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, Lassmann H. Monocyte/macrophage differentiation in early multiple sclerosis lesions. Ann Neurol. 1995 Nov;38(5):788-96.
30. Prineas JW, Kwon EE, Cho ES, Sharer LR, Barnett MH, Oleszak EL, Hoffman B, Morgan BP. Immunopathology of secondary-progressive multiple sclerosis. Ann Neurol. 2001 Nov;50(5):646-57.
31. Hemmer B, Archelos JJ, Hartung HP. New concepts in the immunopathogenesis of multiple sclerosis. Nat Rev Neurosci. 2002 Apr;3(4):291-301.
32. Wucherpfennig KW, Strominger JL. Molecular mimicry in T-cell-mediated autoimmunity: viral peptides activate human T-cell clones specific for myelin basic protein. Cell. 1995 Mar 10;80(5):695-705.
33. Münz C, Lünemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? Nat Rev Immunol. 2009 Apr;9(4):246-58.

34. Furtado GC, Marcondes MC, Latkowski JA, Tsai J, Wensky A, Lafaille JJ. Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis. J Immunol. 2008 Oct 1;181(7):4648-55.
35. Zhang H, Podojil JR, Luo X, Miller SD. Intrinsic and induced regulation of the age-associated onset of spontaneous experimental autoimmune encephalomyelitis. J Immunol. 2008 Oct 1;181(7):4638-47.
36. Dhib-Jalbut S. Pathogenesis of myelin/oligodendrocyte damage in multiple sclerosis. Neurology. 2007 May 29;68(22 Suppl 3):S13-21; discussion S43-54.
37. Schmidt S, Linington C, Zipp F, Sotgiu S, de Waal Malefyt R, Wekerle H, Hohlfeld R. Multiple sclerosis: comparison of the human T-cell response to S100 beta and myelin basic protein reveals parallels to rat experimental autoimmune panencephalitis. Brain. 1997 Aug;120 (Pt 8):1437-45.
38. van Noort JM, van Sechel AC, Bajramovic JJ, el Ouagmiri M, Polman CH, Lassmann H, Ravid R. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. Nature. 1995 Jun 29;375(6534):798-801.
39. Steinman L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Cell. 1996 May 3;85(3):299-302.
40. Noseworthy JH. Progress in determining the causes and treatment of multiple sclerosis. Nature. 1999 Jun 24;399(6738 Suppl):A40-7.
41. Lucchinetti CF, Brueck W, Rodriguez M, Lassmann H. Multiple sclerosis: lessons from neuropathology. Semin Neurol. 1998;18(3):337-49.
42. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485-517.
43. Delgado S, Sheremata WA. The role of CD4+ T-cells in the development of MS. Neurol Res. 2006 Apr;28(3):245-9.
44. Gold R, Lühder F. Interleukin-17--extended features of a key player in multiple sclerosis. Am J Pathol. 2008 Jan;172(1):8-10.
45. Durelli L, Conti L, Clerico M, Boselli D, Contessa G, Ripellino P, Ferrero B, Eid P, Novelli F. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. Ann Neurol. 2009 May;65(5):499-509.
46. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nat Med. 2008 Mar;14(3):337-42.
47. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schröder R, Deckert M, Schmidt S, Ravid R, Rajewsky K. Clonal expansions of CD8(+) T-cells dominate the T-cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med. 2000 Aug 7;192(3):393-404.
48. Skulina C, Schmidt S, Dornmair K, Babbe H, Roers A, Rajewsky K, Wekerle H, Hohlfeld R, Goebels N. Multiple sclerosis: brain-infiltrating CD8+ T-cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc Natl Acad Sci U S A. 2004 Feb 24;101(8):2428-33.

49. Johnson AJ, Suidan GL, McDole J, Pirko I. The CD8 T-cell in multiple sclerosis: suppressor cell or mediator of neuropathology? Int Rev Neurobiol. 2007;79:73-97.
50. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlén C, Goverman J. A pathogenic role for myelin-specific CD8(+) T-cells in a model for multiple sclerosis. J Exp Med. 2001 Sep 3;194(5):669-76.
51. Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Brück W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain. 2000 Jun;123 (Pt 6):1174-83.
52. Medana I, Martinic MA, Wekerle H, Neumann H. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. Am J Pathol. 2001 Sep;159(3):809-15.
53. Saidha S, Eckstein C, Calabresi PA. New and emerging disease modifying therapies for multiple sclerosis. Ann N Y Acad Sci. 2012 Jan;1247:117-37. doi: 10.1111/j.1749-6632.2011.06272.x.
54. Yong VW, Chabot S, Stuve O, Williams G. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. Neurology. 1998 Sep;51(3):682-9.
55. Dhib-Jalbut S, Marks S. Interferon-beta mechanisms of action in multiple sclerosis. Neurology. 2010 Jan 5;74 Suppl 1:S17-24.
56. Zhang X, Markovic-Plese S. Interferon beta inhibits the Th17 cell-mediated autoimmune response in patients with relapsing-remitting multiple sclerosis. Clin Neurol Neurosurg. 2010 Sep;112(7):641-5.
57. Racke MK, Lovett-Racke AE, Karandikar NJ. The mechanism of action of glatiramer acetate treatment in multiple sclerosis. Neurology. 2010 Jan 5;74 Suppl 1:S25-30.
58. Linker RA, Lee DH, Demir S, Wiese S, Kruse N, Siglienti I, Gerhardt E, Neumann H, Sendtner M, Lühder F, Gold R. Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: therapeutic implications in a model of multiple sclerosis. Brain. 2010 Aug;133(Pt 8):2248-63.
59. Fox EJ. Mechanism of action of mitoxantrone. Neurology. 2004 Dec 28;63(12 Suppl 6):S15-8.
60. Brinkmann V. FTY720 (fingolimod) in Multiple Sclerosis: therapeutic effects in the immune and the central nervous system. Br J Pharmacol. 2009 Nov;158(5):1173-82.
61. Sipe JC. Cladribine for multiple sclerosis: review and current status. Expert Rev Neurother. 2005 Nov;5(6):721-7.
62. Beutler E. Cladribine (2-chlorodeoxyadenosine). Lancet. 1992 Oct 17;340(8825):952-6.
63. Hawker K and Kaufman DM. Safe Administration and Monitoring of Monoclonal Antibodies in the Treatment of Multiple Sclerosis. IJMSC. 2007 April;9(Suppl 2):1-24.
64. Comi G. Treatment of multiple sclerosis: role of natalizumab. Neurol Sci. 2009 Oct;30 Suppl 2:S155-8.
65. Stüve O, Gold R, Chan A, Mix E, Zettl U, Kieseier BC. alpha4-Integrin antagonism with natalizumab: effects and adverse effects. J Neurol. 2008 Dec;255 Suppl 6:58-65.

66. Kappos L, Bates D, Edan G, Eraksoy M, Garcia-Merino A, Grigoriadis N, Hartung HP, Havrdová E, Hillert J, Hohlfeld R, Kremenchutzky M, Lyon-Caen O, Miller A, Pozzilli C, Ravnborg M, Saida T, Sindic C, Vass K, Clifford DB, Hauser S, Major EO, O'Connor PW, Weiner HL, Clanet M, Gold R, Hirsch HH, Radü EW, Sørensen PS, King J. Natalizumab treatment for multiple sclerosis: updated recommendations for patient selection and monitoring. Lancet Neurol. 2011 Aug;10(8):745-58.
67. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, Phillips JT, Lublin FD, Giovannoni G, Wajgt A, Toal M, Lynn F, Panzara MA, Sandrock AW; AFFIRM Investigators. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med. 2006 Mar 2;354(9):899-910.
68. Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue EW, Lublin FD, Weinstock-Guttman B, Wynn DR, Lynn F, Panzara MA, Sandrock AW; SENTINEL Investigators. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. N Engl J Med. 2006 Mar 2;354(9):911-23.
69. Langer-Gould A, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. N Engl J Med. 2005 Jul 28;353(4):375-81.
70. Kleinschmidt-DeMasters BK, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. N Engl J Med. 2005 Jul 28;353(4):369-74.
71. Van Assche G, Van Ranst M, Sciot R, Dubois B, Vermeire S, Noman M, Verbeeck J, Geboes K, Robberecht W, Rutgeerts P. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. N Engl J Med. 2005 Jul 28;353(4):362-8.
72. Dearden CE. Alemtuzumab in lymphoproliferate disorders. Rev Clin Exp Hematol. 2002 Dec;6(4):435-48; discussion 449-50.
73. Coles A, Deans J, Compston A. Campath-1H treatment of multiple sclerosis: lessons from the bedside for the bench. Clin Neurol Neurosurg. 2004 Jun;106(3):270-4.
74. Rose JW, Watt HE, White AT, Carlson NG. Treatment of multiple sclerosis with an anti-interleukin-2 receptor monoclonal antibody. Ann Neurol. 2004 Dec;56(6):864-7.
75. Bielekova B, Richert N, Howard T, Blevins G, Markovic-Plese S, McCartin J, Frank JA, Würfel J, Ohayon J, Waldmann TA, McFarland HF, Martin R. Humanized anti-CD25 (daclizumab) inhibits disease activity in multiple sclerosis patients failing to respond to interferon beta. Proc Natl Acad Sci U S A. 2004 Jun 8;101(23):8705-8.
76. Bielekova B, Catalfamo M, Reichert-Scrivner S, Packer A, Cerna M, Waldmann TA, McFarland H, Henkart PA, Martin R. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc Natl Acad Sci U S A. 2006 Apr 11;103(15):5941-6.
77. Vincenti F, Kirkman R, Light S, Bumgardner G, Pescovitz M, Halloran P, Neylan J, Wilkinson A, Ekberg H, Gaston R, Backman L, Burdick J.

Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. N Engl J Med. 1998 Jan 15;338(3):161-5.
78. Arkfeld DG. The potential utility of B-cell-directed biologic therapy in autoimmune diseases. Rheumatol Int. 2008 Jan;28(3):205-15..
79. Rastetter W, Molina A, White CA. Rituximab: expanding role in therapy for lymphomas and autoimmune diseases. Annu Rev Med. 2004;55:477-503.
80. Rose JW, Foley J, Carlson N. Monoclonal antibody treatments for multiple sclerosis. Curr Neurol Neurosci Rep. 2008 Sep;8(5):419-26.
81. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, Langer-Gould A, Smith CH; HERMES Trial Group. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008 Feb 14;358(7):676-88.
82. Buttmann M, Rieckmann P. Treating multiple sclerosis with monoclonal antibodies. Expert Rev Neurother. 2008 Mar;8(3):433-55.
83. Astrom KE, Mancall EL, Richardson EP Jr. Progressive multifocal leuko-encephalopathy; a hitherto unrecognized complication of chronic lymphatic leukaemia and Hodgkin's disease. Brain. 1958 Mar;81(1):93-111.
84. White MK, Khalili K. Pathogenesis of progressive multifocal leukoencephalopathy--revisited. J Infect Dis. 2011 Mar 1;203(5):578-86.
85. Major EO. Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies. Annu Rev Med. 2010;61:35-47.
86. Power C, Gladden JG, Halliday W, Del Bigio MR, Nath A, Ni W, Major EO, Blanchard J, Mowat M. AIDS- and non-AIDS-related PML association with distinct p53 polymorphism. Neurology. 2000 Feb 8;54(3):743-6.
87. Tan CS, Koralnik IJ. Beyond progressive multifocal leukoencephalopathy: expanded pathogenesis of JC virus infection in the central nervous system. Lancet Neurol. 2010 April ; 9(4): 425–437. doi:10.1016/S1474-4422(10)70040-5.
88. Clifford DB, Yiannoutsos C, Glicksman M, Simpson DM, Singer EJ, Piliero PJ, Marra CM, Francis GS, McArthur JC, Tyler KL, Tselis AC, Hyslop NE. HAART improves prognosis in HIV-associated progressive multifocal leukoencephalopathy. Neurology. 1999 Feb;52(3):623-5.
89. Cinque P, Koralnik IJ, Gerevini S, Miro JM, Price RW. Progressive multifocal leukoencephalopathy in HIV-1 infection. Lancet Infect Dis. 2009 Oct;9(10):625-36.
90. Tavazzi E, White MK, Khalili K. Progressive multifocal leukoencephalopathy: clinical and molecular aspects. Rev Med Virol. 2012 Jan;22(1):18-32. doi: 10.1002/rmv.710.
91. Berenguer J, Miralles P, Arrizabalaga J, Ribera E, Dronda F, Baraia-Etxaburu J, Domingo P, Márquez M, Rodriguez-Arrondo FJ, Laguna F, Rubio R, Lacruz Rodrigo J, Mallolas J, de Miguel V; GESIDA 11/99 Study Group. Clinical course and prognostic factors of progressive multifocal leukoencephalopathy in patients treated with highly active antiretroviral therapy. Clin Infect Dis. 2003 Apr 15;36(8):1047-52.

92. Molloy ES, Calabrese LH. Progressive multifocal leukoencephalopathy: a national estimate of frequency in systemic lupus erythematosus and other rheumatic diseases. Arthritis Rheum. 2009 Dec;60(12):3761-5.
93. Berger JR, Pall L, Lanska D, Whiteman M. Progressive multifocal leukoencephalopathy in patients with HIV infection. J Neurovirol. 1998 Feb;4(1):59-68.
94. Guilleux MH, Steiner RE, Young IR. MR imaging in progressive multifocal leukoencephalopathy. AJNR Am J Neuroradiol. 1986 Nov-Dec;7(6):1033-5.
95. Wiley CA, Grafe M, Kennedy C, Nelson JA. Human immunodeficiency virus (HIV) and JC virus in acquired immune deficiency syndrome (AIDS) patients with progressive multifocal leukoencephalopathy. Acta Neuropathol. 1988;76(4):338-46.
96. Del Valle L, Piña-Oviedo S. HIV disorders of the brain: pathology and pathogenesis. Front Biosci. 2006 Jan 1;11:718-32.
97. Kuchelmeister K, Gullotta F, Bergmann M, Angeli G, Masini T. Progressive multifocal leukoencephalopathy (PML) in the acquired immunodeficiency syndrome (AIDS). A neuropathological autopsy study of 21 cases. Pathol Res Pract. 1993 Mar;189(2):163-73.
98. Aksamit AJ, Gendelman HE, Orenstein JM, Pezeshkpour GH. AIDS-associated progressive multifocal leukoencephalopathy (PML): comparison to non-AIDS PML with in situ hybridization and immunohistochemistry. Neurology. 1990 Jul;40(7):1073-8.
99. Vendrely A, et al. 2005. Fulminant inflammatory leukoencephalopathy associated with HAART-induced immune restoration in AIDS-related progressive multifocal leukoencephalopathy. Acta Neuropathol. 109:449–455.
100. Wuthrich C, et al. 2006. Characterization of lymphocytic infiltrates in progressive multifocal leukoencephalopathy: co-localization of CD8(+) T-cells with JCV-infected glial cells. J. Neurovirol. 12:116 –128.
101. Berger JR. Progressive multifocal leukoencephalopathy and newer biological agents. Drug Saf. 2010 Nov 1;33(11):969-83. doi: 10.2165/11537510-000000000-00000.
102. Delbue S, Marchioni E, Sotgiu G, Saresella M, Tavazzi E, Colombo E, Guerini FR, Maserati R, Sinforiani E, Schifino MR, Ferrante P. Longitudinal study of two cases of progressive multifocal leukoencephalopathy with a clinical benign evolution. J Neurovirol. 2007 Jun;13(3):268-73.
103. Berger JR, Kaszovitz B, Post MJ, Dickinson G. Progressive multifocal leukoencephalopathy associated with human immunodeficiency virus infection. A review of the literature with a report of sixteen cases. Ann Intern Med. 1987 Jul;107(1):78-87.
104. Cinque P, Koralnik IJ, Clifford DB. The evolving face of human immunodeficiency virus-related progressive multifocal leukoencephalopathy: defining a consensus terminology. J Neurovirol. 2003;9 Suppl 1:88-92.
105. Koralnik IJ, Boden D, Mai VX, Lord CI, Letvin NL. JC virus DNA load in patients with and without progressive multifocal leukoencephalopathy. Neurology. 1999 Jan 15;52(2):253-60.

106. Marzocchetti A, Di Giambenedetto S, Cingolani A, Ammassari A, Cauda R, De Luca A. Reduced rate of diagnostic positive detection of JC virus DNA in cerebrospinal fluid in cases of suspected progressive multifocal leukoencephalopathy in the era of potent antiretroviral therapy. J Clin Microbiol. 2005 Aug;43(8):4175-7.
107. Tan CS, Koralnik IJ. Progressive multifocal leukoencephalopathy and other disorders caused by JC virus: clinical features and pathogenesis. Lancet Neurol. 2010 Apr;9(4):425-37.
108. Warnke C, Menge T, Hartung HP, Racke MK, Cravens PD, Bennett JL, Frohman EM, Greenberg BM, Zamvil SS, Gold R, Hemmer B, Kieseier BC, Stüve O. Natalizumab and progressive multifocal leukoencephalopathy: what are the causal factors and can it be avoided? Arch Neurol. 2010 Aug;67(8):923-30.
109. Cinque P, Bossolasco S, Brambilla AM, Boschini A, Mussini C, Pierotti C, Campi A, Casari S, Bertelli D, Mena M, Lazzarin A. The effect of highly active antiretroviral therapy-induced immune reconstitution on development and outcome of progressive multifocal leukoencephalopathy: study of 43 cases with review of the literature. J Neurovirol. 2003;9 Suppl 1:73-80.
110. Brew BJ, Davies NW, Cinque P, Clifford DB, Nath A. Progressive multifocal leukoencephalopathy and other forms of JC virus disease. Nat Rev Neurol. 2010 Dec;6(12):667-79.
111. Andrei G, Snoeck R, Vandeputte M, De Clercq E. Activities of various compounds against murine and primate polyomaviruses. Antimicrob Agents Chemother. 1997 Mar;41(3):587-93.
112. Hou J, Major EO. The efficacy of nucleoside analogs against JC virus multiplication in a persistently infected human fetal brain cell line. J Neurovirol. 1998 Aug;4(4):451-6.
113. Pho MT, Ashok A, Atwood WJ. JC virus enters human glial cells by clathrin-dependent receptor-mediated endocytosis. J Virol. 2000 Mar;74(5):2288-92.
114. Brickelmaier M, Lugovskoy A, Kartikeyan R, Reviriego-Mendoza MM, Allaire N, Simon K, Frisque RJ, Gorelik L. Identification and characterization of mefloquine efficacy against JC virus in vitro. Antimicrob Agents Chemother. 2009 May;53(5):1840-9.
115. Toovey S. Mefloquine neurotoxicity: a literature review. Travel Med Infect Dis. 2009 Jan;7(1):2-6.
116. Gofton TE, Al-Khotani A, O'Farrell B, Ang LC, McLachlan RS. Mefloquine in the treatment of progressive multifocal leukoencephalopathy. J Neurol Neurosurg Psychiatry. 2011 Apr;82(4):452-5.
117. Friedman R. News from the AAN Annual Meeting: Malaria Drug Fails to Fulfill Promise in PML. Neurology Today 2011 April;11(8); p 8.
118. Johnson T, Nath A. Immune reconstitution inflammatory syndrome and the central nervous system. Curr Opin Neurol. 2011 Jun;24(3):284-90.
119. Ferenczy MW, Marshall LJ, Nelson CD, Atwood WJ, Nath A, Khalili K, Major EO. Molecular biology, epidemiology, and pathogenesis of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease

of the human brain. Clin Microbiol Rev. 2012 Jul;25(3):471-506. doi: 10.1128/CMR.05031-11.
120. Gasnault J, Costagliola D, Hendel-Chavez H, Dulioust A, Pakianather S, Mazet AA, de Goer de Herve MG, Lancar R, Lascaux AS, Porte L, Delfraissy JF, Taoufik Y; ANRS 125 Trial Team. Improved survival of HIV-1-infected patients with progressive multifocal leukoencephalopathy receiving early 5-drug combination antiretroviral therapy. PLoS One. 2011;6(6):e20967.
121. Marzocchetti A, Tompkins T, Clifford DB, Gandhi RT, Kesari S, Berger JR, Simpson DM, Prosperi M, De Luca A, Koralnik IJ. Determinants of survival in progressive multifocal leukoencephalopathy. Neurology. 2009 Nov 10;73(19):1551-8.
122. Berger JR, Levy RM, Flomenhoft D, Dobbs M. Predictive factors for prolonged survival in acquired immunodeficiency syndrome-associated progressive multifocal leukoencephalopathy. Ann Neurol. 1998 Sep;44(3):341-9.
123. Taoufik Y, Gasnault J, Karaterki A, Pierre Ferey M, Marchadier E, Goujard C, Lannuzel A, Delfraissy JF, Dussaix E. Prognostic value of JC virus load in cerebrospinal fluid of patients with progressive multifocal leukoencephalopathy. J Infect Dis. 1998 Dec;178(6):1816-20.
124. Yiannoutsos CT, Major EO, Curfman B, Jensen PN, Gravell M, Hou J, Clifford DB, Hall CD. Relation of JC virus DNA in the cerebrospinal fluid to survival in acquired immunodeficiency syndrome patients with biopsy-proven progressive multifocal leukoencephalopathy. Ann Neurol. 1999 Jun;45(6):816-21.
125. Antinori A, Cingolani A, Lorenzini P, Giancola ML, Uccella I, Bossolasco S, Grisetti S, Moretti F, Vigo B, Bongiovanni M, Del Grosso B, Arcidiacono MI, Fibbia GC, Mena M, Finazzi MG, Guaraldi G, Ammassari A, d'Arminio Monforte A, Cinque P, De Luca A; Italian Registry Investigative Neuro AIDS Study Group. Clinical epidemiology and survival of progressive multifocal leukoencephalopathy in the era of highly active antiretroviral therapy: data from the Italian Registry Investigative Neuro AIDS (IRINA). J Neurovirol. 2003;9 Suppl 1:47-53.
126. French MA, Price P, Stone SF. Immune restoration disease after antiretroviral therapy. AIDS. 2004 Aug 20;18(12):1615-27.
127. French MA. HIV/AIDS: immune reconstitution inflammatory syndrome: a reappraisal. Clin Infect Dis. 2009 Jan 1;48(1):101-7.
128. Vendrely A, Bienvenu B, Gasnault J, Thiebault JB, Salmon D, Gray F. Fulminant inflammatory leukoencephalopathy associated with HAART-induced immune restoration in AIDS-related progressive multifocal leukoencephalopathy. Acta Neuropathol. 2005 Apr;109(4):449-55.
129. Weissert R. Progressive multifocal leukoencephalopathy. J Neuroimmunol. 2011 Feb;231(1-2):73-7.
130. Berger JR. Progressive multifocal leukoencephalopathy in acquired immunodeficiency syndrome: explaining the high incidence and disproportionate frequency of the illness relative to other immunosuppressive conditions. J Neurovirol. 2003;9 Suppl 1:38-41.

131. Gasnault J, Kahraman M, de Goër de Herve MG, Durali D, Delfraissy JF, Taoufik Y. Critical role of JC virus-specific CD4 T-cell responses in preventing progressive multifocal leukoencephalopathy. AIDS. 2003 Jul 4;17(10):1443-9.
132. Houff SA, Berger JR. The bone marrow, B-cells, and JC virus. J Neurovirol. 2008 Oct;14(5):341-3.
133. Chowdhury M, Taylor JP, Tada H, Rappaport J, Wong-Staal F, Amini S, Khalili K. Regulation of the human neurotropic virus promoter by JCV-T antigen and HIV-1 tat protein. Oncogene. 1990 Dec;5(12):1737-42.
134. Kaniowska D, Kaminski R, Amini S, Radhakrishnan S, Rappaport J, Johnson E, Khalili K, Del Valle L, Darbinyan A. Cross-interaction between JC virus agnoprotein and human immunodeficiency virus type 1 (HIV-1) Tat modulates transcription of the HIV-1 long terminal repeat in glial cells. J Virol. 2006 Sep;80(18):9288-99.
135. Khalili K, White MK. Human demyelinating disease and the polyomavirus JCV. Mult Scler. 2006 Apr;12(2):133-42.
136. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell. 1988 Dec 23;55(6):1189-93.
137. Ault GS. Activity of JC virus archetype and PML-type regulatory regions in glial cells. J Gen Virol. 1997 Jan;78 (Pt 1):163-9.
138. Li W, Li G, Steiner J, Nath A. Role of Tat protein in HIV neuropathogenesis. Neurotox Res. 2009 Oct;16(3):205-20.
139. Vincendeau M, Kramer S, Hadian K, Rothenaigner I, Bell J, Hauck SM, Bickel C, Nagel D, Kremmer E, Werner T, Leib-Mösch C, Brack-Werner R. Control of HIV replication in astrocytes by a family of highly conserved host proteins with a common Rev-interacting domain (Risp). AIDS. 2010 Oct 23;24(16):2433-42.
140. Leonardi CL. Current concepts and review of efalizumab in the treatment of psoriasis. Dermatol Clin. 2004 Oct;22(4):427-35, ix.
141. Lebwohl M, Tyring SK, Hamilton TK, Toth D, Glazer S, Tawfik NH, Walicke P, Dummer W, Wang X, Garovoy MR, Pariser D; Efalizumab Study Group. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. N Engl J Med. 2003 Nov 20;349(21):2004-13.
142. Gürcan HM, Keskin DB, Stern JN, Nitzberg MA, Shekhani H, Ahmed AR. A review of the current use of rituximab in autoimmune diseases. Int Immunopharmacol. 2009 Jan;9(1):10-25.
143. Carson KR, Evens AM, Richey EA, Habermann TM, Focosi D, Seymour JF, Laubach J, Bawn SD, Gordon LI, Winter JN, Furman RR, Vose JM, Zelenetz AD, Mamtani R, Raisch DW, Dorshimer GW, Rosen ST, Muro K, Gottardi-Littell NR, Talley RL, Sartor O, Green D, Major EO, Bennett CL. Progressive multifocal leukoencephalopathy after rituximab therapy in HIV-negative patients: a report of 57 cases from the Research on Adverse Drug Events and Reports project. Blood. 2009 May 14;113(20):4834-40. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, Langer-Gould A, Smith CH; HERMES Trial Group. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008 Feb 14;358(7):676-88.

144. Tavazzi E, Ferrante P, Khalili K. Progressive multifocal leukoencephalopathy: an unexpected complication of modern therapeutic monoclonal antibody therapies. Clin Microbiol Infect. 2011 Dec;17(12):1776-80. doi: 10.1111/j.1469-0691.2011.03653.x.
145. Budka H, Wiley CA, Kleihues P, Artigas J, Asbury AK, Cho ES, Cornblath DR, Dal Canto MC, DeGirolami U, Dickson D, et al. HIV-associated disease of the nervous system: review of nomenclature and proposal for neuropathology-based terminology. Brain Pathol. 1991 Apr;1(3):143-52.
146. Langford TD, Letendre SL, Marcotte TD, Ellis RJ, McCutchan JA, Grant I, Mallory ME, Hansen LA, Archibald S, Jernigan T, Masliah E; HNRC Group. Severe, demyelinating leukoencephalopathy in AIDS patients on antiretroviral therapy. AIDS. 2002 May 3;16(7):1019-29.
147. Eddy BE, Stewart SE, Berkeley W. Cytopathogenicity in tissue culture by a tumor virus from mice. Proc Soc Exp Biol Med. 1958 Aug-Sep;98(4):848-51.
148. Gjoerup O, Chang Y. Update on human polyomaviruses and cancer. Adv Cancer Res. 2010;106:1-51.
149. Imperiale MJ. The human polyomaviruses: an overview. In: Human polyomavirus molecular and clinical perspectives. Khalili K, Stoner GL, eds. John Wiley & Sons, Inc Publication. Ch. 5, pp. 53-71, 2001.
150. Eash S, Manley K, Gasparovic M, Querbes W, Atwood WJ. The human polyomaviruses. Cell Mol Life Sci. 2006 Apr;63(7-8):865-76.
151. Gardner SD, Field AM, Coleman DV, Hulme B. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet. 1971 Jun 19;1(7712):1253-7.
152. Padgett BL, Walker DL, ZuRhein GM, Eckroade RJ, Dessel BH. Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. Lancet. 1971 Jun 19;1(7712):1257-60.
153. Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. Identification of a third human polyomavirus. J Virol. 2007 Apr;81(8):4130-6.
154. Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. 2007 May 4;3(5):e64.
155. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008 Feb 22;319(5866):1096-100.
156. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe. 2010 Jun 25;7(6):509-15.
157. van der Meijden E, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MC. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. PLoS Pathog. 2010 Jul 29;6(7):e1001024.
158. Scuda N, Hofmann J, Calvignac-Spencer S, Ruprecht K, Liman P, Kühn J, Hengel H, Ehlers B. A novel human polyomavirus closely related to the african

green monkey-derived lymphotropic polyomavirus. J Virol. 2011 May;85(9):4586-90.
159. Buck CB, Phan GQ, Raiji MT, Murphy PM, McDermott DH, McBride AA. Complete genome sequence of a tenth human polyomavirus. J Virol. 2012 Oct;86(19):10887. doi: 10.1128/JVI.01690-12.
160. Knowles WA. Discovery and epidemiology of the human polyomaviruses BK virus (BKV) and JC virus (JCV). Adv Exp Med Biol. 2006;577:19-45.
161. Doerries K. Human polyomavirus JC and BK persistent infection. Adv Exp Med Biol. 2006;577:102-16.
162. Van Ghelue M, Khan MT, Ehlers B, Moens U. Genome analysis of the new human polyomaviruses. Rev Med Virol. 2012 Nov;22(6):354-77. doi: 10.1002/rmv.1711.
163. Ahsan N, Shah KV. Polyomaviruses and human diseases. Adv Exp Med Biol. 2006;577:1-18.
164. Moens U and Rekvig OP, Molecular biology of BK virus and clinical and basic aspects of BK virus renal infection. In: human Polyomaviruses: molecular and clinical perspectives. Pp 359-408.
165. Lee W, Langhoff E. Polyomavirus in human cancer development. Adv Exp Med Biol. 2006;577:310-8.
166. Trowbridge PW, Frisque RJ. Identification of three new JC virus proteins generated by alternative splicing of the early viral mRNA. J Neurovirol. 1995 Jun;1(2):195-206.
167. Abend JR, Joseph AE, Das D, Campbell-Cecen DB, Imperiale MJ. A truncated T antigen expressed from an alternatively spliced BK virus early mRNA. J Gen Virol. 2009 May;90(Pt 5):1238-45.
168. Khalili K, White MK, Sawa H, Nagashima K, Safak M. The agnoprotein of polyomaviruses: a multifunctional auxiliary protein. J Cell Physiol. 2005 Jul;204(1):1-7.
169. Liu CK, Wei G, Atwood WJ. Infection of glial cells by the human polyomavirus JC is mediated by an N-linked glycoprotein containing terminal alpha(2-6)-linked sialic acids. J Virol. 1998 Jun;72(6):4643-9.
170. Neu U, Maginnis MS, Palma AS, Ströh LJ, Nelson CD, Feizi T, Atwood WJ, Stehle T. Structure-function analysis of the human JC polyomavirus establishes the LSTc pentasaccharide as a functional receptor motif. Cell Host Microbe. 2010 Oct 21;8(4):309-19.
171. Elphick GF, Querbes W, Jordan JA, Gee GV, Eash S, Manley K, Dugan A, Stanifer M, Bhatnagar A, Kroeze WK, Roth BL, Atwood WJ. The human polyomavirus, JCV, uses serotonin receptors to infecT-cells. Science. 2004 Nov 19;306(5700):1380-3.
172. Maginnis MS, Haley SA, Gee GV, Atwood WJ. Role of N-linked glycosylation of the 5-HT2A receptor in JC virus infection. J Virol. 2010 Oct;84(19):9677-84.
173. Cole CN, Conzen SD. Polyomavirinae: the viruses and their replication. In: Fundamental virology, 3rd ed. Knipe DM, Howley PM, eds. Lippincott Williams and Wilkins, Philadelphia. 2001:vol.1, pp. 985-1018.

174. Khalili K, Gordon J, White MK. The polyomavirus, JCV and its involvement in human disease. Adv Exp Med Biol. 2006;577:274-87.
175. Monaco MC, Jensen PN, Hou J, Durham LC, Major EO. Detection of JC virus DNA in human tonsil tissue: evidence for site of initial viral infection. J Virol. 1998 Dec;72(12):9918-23.
176. Bofill-Mas S, Girones R. Excretion and transmission of JCV in human populations. J Neurovirol. 2001 Aug;7(4):345-9.
177. Bofill-Mas S, Clemente-Casares P, Major EO, Curfman B, Girones R. Analysis of the excreted JC virus strains and their potential oral transmission. J Neurovirol. 2003 Aug;9(4):498-507.
178. Bofill-Mas S, Formiga-Cruz M, Clemente-Casares P, Calafell F, Girones R. Potential Transmission of Human Polyomaviruses through the Gastrointestinal Tract after Exposure to Virions or Viral DNA. J Virol. 2001 November; 75(21): 10290–10299. doi: 10.1128/JVI.75.21.10290-10299.2001
179. Ricciardiello L, Laghi L, Ramamirtham P, Chang CL, Chang DK, Randolph AE, Boland CR. JC virus DNA sequences are frequently present in the human upper and lower gastrointestinal tract. Gastroenterology. 2000 Nov;119(5):1228-35.
180. Laghi L,Randolph AE, Chauhan DP, Marra G, Major EO, Neel JV, Boland CR. JC virus DNA is present in the mucosa of the human colon and in colorectal cancers. Proc Natl Acad Sci U S A. 1999 June 22; 96(13): 7484–7489.
181. Jiang M, Abend JR, Johnson SF, Imperiale MJ. The role of polyomaviruses in human disease. Virology. 2009 Feb 20;384(2):266-73.
182. Berger JR, Concha M. Progressive multifocal leukoencephalopathy: the evolution of a disease once considered rare. J Neurovirol. 1995 Mar;1(1):5-18.
183. Brooks BR, Walker DL. Progressive multifocal leukoencephalopathy. Neurol Clin. 1984 May;2(2):299-313.
184. Frisque RJ, Bream GL, Cannella MT. Human polyomavirus JC virus genome. J Virol. 1984 Aug;51(2):458-69.
185. Jensen PN, Major EO. A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region. J Neurovirol. 2001 Aug;7(4):280-7.
186. Yogo Y, Kitamura T, Sugimoto C, Ueki T, Aso Y, Hara K, Taguchi F. Isolation of a possible archetypal JC virus DNA sequence from nonimmunocompromised individuals. J Virol. 1990 Jun;64(6):3139-43.
187. Marshall LJ, Dunham L, Major EO. Transcription factor Spi-B binds unique sequences present in the tandem repeat promoter/enhancer of JC virus and supports viral activity. J Gen Virol. 2010 Dec;91(Pt 12):3042-52.
188. Cui X, Wang JC, Deckhut A, Joseph BC, Eberwein P, Cubitt CL, Ryschkewitsch CF, Agostini HT, Stoner GL. Chinese strains (Type 7) of JC virus are afro-asiatic in origin but are phylogenetically distinct from the Mongolian and Indian strains (Type 2D) and the Korean and Japanese strains (Type 2A). J Mol Evol. 2004 May;58(5):568-83.
189. Yanagihara R, Nerurkar VR, Scheirich I, Agostini HT, Mgone CS, Cui X, Jobes DV, Cubitt CL, Ryschkewitsch CF, Hrdy DB, Friedlaender JS, Stoner

GL. JC virus genotypes in the western Pacific suggest Asian mainland relationships and virus association with early population movements. Hum Biol. 2002 Jun;74(3):473-88.
190. Jobe DV, Friedlaender JS, Mgone CS, Agostini HT, Koki G, Yanagihara R, Ng TCN, Chima SC, Ryschkewitsch CF, Stoner GL. New JC virus (JCV) genotypes from papua new guinea and micronesia (type 8 and type 2E) and evolutionary analysis of 32 complete JCV genomes. Arch Virol. 2001;146(11):2097-113.
191. Agostini HT, Ryschkewitsch CF, Singer EJ, Baumhefner RW, Stoner GL. JC virus type 2B is found more frequently in brain tissue of progressive multifocal leukoencephalopathy patients than in urine from controls. J Hum Virol. 1998 Mar-Apr;1(3):200-6.
192. Major EO, Miller AE, Mourrain P, Traub RG, de Widt E, Sever J. Establishment of a line of human fetal glial cells that supports JC virus multiplication. Proc Natl Acad Sci U S A. 1985 Feb;82(4):1257-61.
193. Hara K, Sugimoto C, Kitamura T, Aoki N, Taguchi F, Yogo Y. Archetype JC virus efficiently replicates in COS-7 cells, simian cells constitutively expressing simian virus 40 T antigen. J Virol. 1998 Jul;72(7):5335-42.
194. Lynch KJ, Frisque RJ. Factors contributing to the restricted DNA replicating activity of JC virus. Virology. 1991 Jan;180(1):306-17.
195. Tada H, Lashgari M, Rappaport J, Khalili K. Cell type-specific expression of JC virus early promoter is determined by positive and negative regulation. J Virol. 1989 Jan;63(1):463-6.
196. Kenney S, Natarajan V, Strike D, Khoury G, Salzman NP. JC virus enhancer-promoter active in human brain cells. Science. 1984 Dec 14;226(4680):1337-9.
197. Trapp BD, Small JA, Pulley M, Khoury G, Scangos GA. Dysmyelination in transgenic mice containing JC virus early region. Ann Neurol. 1988 Jan;23(1):38-48.
198. Feigenbaum L, Hinrichs SH, Jay G. JC virus and simian virus 40 enhancers and transforming proteins: role in determining tissue specificity and pathogenicity in transgenic mice. J Virol. 1992 Feb;66(2):1176-82.
199. Ahmed S, Chowdhury M, Khalili K. Regulation of a human neurotropic virus promoter, JCVE: identification of a novel activator domain located upstream from the 98 bp enhancer promoter region. Nucleic Acids Res. 1990 Dec 25;18(24):7417-23.
200. Ahmed S, Rappaport J, Tada H, Kerr D, Khalili K. A nuclear protein derived from brain cells stimulates transcription of the human neurotropic virus promoter, JCVE, in vitro. J Biol Chem. 1990 Aug 15;265(23):13899-905.
201. White MK, Gordon J, Reiss K, Del Valle L, Croul S, Giordano A, Darbinyan A, Khalili K. Human polyomaviruses and brain tumors. Brain Res Brain Res Rev. 2005 Dec 1;50(1):69-85.
202. Khalili K, Del Valle L, Otte J, Weaver M, Gordon J. Human neurotropic polyomavirus, JCV, and its role in carcinogenesis. Oncogene. 2003 Aug 11;22(33):5181-91.

203. Major EO. History and current concepts in the pathogenesis of PML. Cleve Clin J Med. 2011 Nov;78 Suppl 2:S3-7.
204. Zurhein G, Chou SM. Particles resembling papova viruses in human cerebral demyelinating disease. Science. 1965 Jun 11;148(3676):1477-9.
205. Mateen FJ, Muralidharan R, Carone M, van de Beek D, Harrison DM, Aksamit AJ, Gould MS, Clifford DB, Nath A. Progressive multifocal leukoencephalopathy in transplant recipients. Ann Neurol. 2011 Aug;70(2):305-22. doi: 10.1002/ana.22408.
206. Randhawa P, Vats A, Shapiro R. The pathobiology of polyomavirus infection in man. Adv Exp Med Biol. 2006;577:148-59.
207. Perez-Liz G, Del Valle L, Gentilella A, Croul S, Khalili K. Detection of JC virus DNA fragments but not proteins in normal brain tissue. Ann Neurol. 2008 Oct;64(4):379-87.
208. Delbue S, Branchetti E, Boldorini R, Vago L, Zerbi P, Veggiani C, Tremolada S, Ferrante P. Presence and expression of JCV early gene large T Antigen in the brains of immunocompromised and immunocompetent individuals. J Med Virol. 2008 Dec;80(12):2147-52.
209. Berger JR, Houff SA, Major EO. Monoclonal antibodies and progressive multifocal leukoencephalopathy. MAbs. 2009 Nov-Dec;1(6):583-9.
210. Tan CS, Dezube BJ, Bhargava P, Autissier P, Wüthrich C, Miller J, Koralnik IJ. Detection of JC virus DNA and proteins in the bone marrow of HIV-positive and HIV-negative patients: implications for viral latency and neurotropic transformation. J Infect Dis. 2009 Mar 15;199(6):881-8.
211. Tornatore C, Berger JR, Houff SA, Curfman B, Meyers K, Winfield D, Major EO. Detection of JC virus DNA in peripheral lymphocytes from patients with and without progressive multifocal leukoencephalopathy. Ann Neurol. 1992 Apr;31(4):454-62.
212. Chapagain ML, Verma S, Mercier F, Yanagihara R, Nerurkar VR. Polyomavirus JC infects human brain microvascular endothelial cells independent of serotonin receptor 2A. Virology. 2007 Jul 20;364(1):55-63.
213. Richardson EP Jr, Webster HD. Progressive multifocal leukoencephalopathy: its pathological features. Prog Clin Biol Res. 1983;105:191-203.
214. Kuchelmeister K, Bergmann M, Gullotta F. Cellular changes in the cerebellar granular layer in AIDS-associated PML. Neuropathol Appl Neurobiol. 1993 Oct;19(5):398-401.
215. Tagliati M, Simpson D, Morgello S, Clifford D, Schwartz RL, Berger JR. Cerebellar degeneration associated with human immunodeficiency virus infection. Neurology. 1998 Jan;50(1):244-51.
216. Wüthrich C, Dang X, Westmoreland S, McKay J, Maheshwari A, Anderson MP, Ropper AH, Viscidi RP, Koralnik IJ. Fulminant JC virus encephalopathy with productive infection of cortical pyramidal neurons. Ann Neurol. 2009 Jun;65(6):742-8.
217. Behzad-Behbahani A, Klapper PE, Vallely PJ, Cleator GM, Bonington A. BKV-DNA and JCV-DNA in CSF of patients with suspected meningitis or encephalitis. Infection. 2003 Dec;31(6):374-8.

218. Viallard JF, Ellie E, Lazaro E, Lafon ME, Pellegrin JL. JC virus meningitis in a patient with systemic lupus erythematosus. Lupus. 2005;14(12):964-6.
219. Blake K, Pillay D, Knowles W, Brown DW, Griffiths PD, Taylor B. JC virus associated meningoencephalitis in an immunocompetent girl. Arch Dis Child. 1992 Jul;67(7):956-7.
220. Kleinschmidt-DeMasters BK, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. N Engl J Med. 2005 Jul 28;353(4):369-74.
221. Langer-Gould A, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. N Engl J Med. 2005 Jul 28;353(4):375-81.
222. Van Assche G, Van Ranst M, Sciot R, Dubois B, Vermeire S, Noman M, Verbeeck J, Geboes K, Robberecht W, Rutgeerts P. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. N Engl J Med. 2005 Jul 28;353(4):362-8.
223. Yousry TA, Major EO, Ryschkewitsch C, Fahle G, Fischer S, Hou J, Curfman B, Miszkiel K, Mueller-Lenke N, Sanchez E, Barkhof F, Radue EW, Jäger HR, Clifford DB. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. N Engl J Med. 2006 Mar 2;354(9):924-33.
224. Bloomgren G, Richman S, Hotermans C, Subramanyam M, Goelz S, Natarajan A, Lee S, Plavina T, Scanlon JV, Sandrock A, Bozic C. Risk of natalizumab-associated progressive multifocal leukoencephalopathy. N Engl J Med. 2012 May 17;366(20):1870-80.
225. Stüve O, Marra CM, Jerome KR, Cook L, Cravens PD, Cepok S, Frohman EM, Phillips JT, Arendt G, Hemmer B, Monson NL, Racke MK. Immune surveillance in multiple sclerosis patients treated with natalizumab. Ann Neurol. 2006 May;59(5):743-7.
226. Wüthrich C, Kesari S, Kim WK, Williams K, Gelman R, Elmeric D, De Girolami U, Joseph JT, Hedley-Whyte T, Koralnik IJ. Characterization of lymphocytic infiltrates in progressive multifocal leukoencephalopathy: co-localization of CD8(+) T-cells with JCV-infected glial cells. J Neurovirol. 2006 Apr;12(2):116-28.
227. Du Pasquier RA, Stein MC, Lima MA, Dang X, Jean-Jacques J, Zheng Y, Letvin NL, Koralnik IJ. JC virus induces a vigorous CD8+ cytotoxic T-cell response in multiple sclerosis patients. J Neuroimmunol. 2006 Jul;176(1-2):181-6.
228. Lindå H, von Heijne A, Major EO, Ryschkewitsch C, Berg J, Olsson T, Martin C. Progressive multifocal leukoencephalopathy after natalizumab monotherapy. N Engl J Med. 2009 Sep 10;361(11):1081-7.
229. Chen Y et al., Asymptomatic reactivation of JCV virus in patients treated with Natalizumab. N Engl J Med, 2009
230. Clifford DB, De Luca A, Simpson DM, Arendt G, Giovannoni G, Nath A. Natalizumab-associated progressive multifocal leukoencephalopathy in patients with multiple sclerosis: lessons from 28 cases. Lancet Neurol. 2010 Apr;9(4):438-46.

231. Khatri BO, Man S, Giovannoni G, Koo AP, Lee JC, Tucky B, Lynn F, Jurgensen S, Woodworth J, Goelz S, Duda PW, Panzara MA, Ransohoff RM, Fox RJ. Effect of plasma exchange in accelerating natalizumab clearance and restoring leukocyte function. Neurology. 2009 Feb 3;72(5):402-9.
232. Vermersch P, Kappos L, Gold R, Foley JF, Olsson T, Cadavid D, Bozic C, Richman S. Clinical outcomes of natalizumab-associated progressive multifocal leukoencephalopathy. Neurology. 2011 May 17;76(20):1697-704.
233. Hunt D, Giovannoni G. Natalizumab-associated progressive multifocal leucoencephalopathy: a practical approach to risk profiling and monitoring. Pract Neurol. 2012 Feb;12(1):25-35.
234. Sørensen PS, Bertolotto A, Edan G, Giovannoni G, Gold R, Havrdova E, Kappos L, Kieseier BC, Montalban X, Olsson T. Risk stratification for progressive multifocal leukoencephalopathy in patients treated with natalizumab. Mult Scler. 2012 Feb;18(2):143-52.
235. Kappos L, Bates D, Edan G, Eraksoy M, Garcia-Merino A, Grigoriadis N, Hartung HP, Havrdová E, Hillert J, Hohlfeld R, Kremenchutzky M, Lyon-Caen O, Miller A, Pozzilli C, Ravnborg M, Saida T, Sindic C, Vass K, Clifford DB, Hauser S, Major EO, O'Connor PW, Weiner HL, Clanet M, Gold R, Hirsch HH, Radü EW, Sørensen PS, King J. Natalizumab treatment for multiple sclerosis: updated recommendations for patient selection and monitoring. Lancet Neurol. 2011 Aug;10(8):745-58.
236. Sandrock A, Hotermans C, Richman S, Natarajan A, Lee S, Plavina T, ET AL., Risk stratiofication for progressive multifocal leukoencephalopathy in multiple sclerosis patients using natalizumab. J Neurol. 2011;258:S23.
237. Kappos L, Bates D, Hartung HP, Havrdova E, Miller D, Polman CH, Ravnborg M, Hauser SL, Rudick RA, Weiner HL, O'Connor PW, King J, Radue EW, Yousry T, Major EO, Clifford DB. Natalizumab treatment for multiple sclerosis: recommendations for patient selection and monitoring. Lancet Neurol. 2007 May;6(5):431-41.
238. Gorelik L, Lerner M, Bixler S, Crossman M, Schlain B, Simon K, Pace A, Cheung A, Chen LL, Berman M, Zein F, Wilson E, Yednock T, Sandrock A, Goelz SE, Subramanyam M. Anti-JC virus antibodies: implications for PML risk stratification. Ann Neurol. 2010 Sep;68(3):295-303. doi: 10.1002/ana.22128.
239. Bloomgren G, Richman S, Hotermans C, Subramanyam M, Goelz S, Natarajan A, Lee S, Plavina T, Scanlon JV, Sandrock A, Bozic C. Risk of natalizumab-associated progressive multifocal leukoencephalopathy. N Engl J Med. 2012 May 17;366(20):1870-80.
240. http://www.fda.gov/Drugs/DrugSafety/ucm288186.htm, accessed on November, 9 2012.
241. Rudick RA, O'Connor PW, Polman CH, Goodman AD, Ray SS, Griffith NM, Jurgensen SA, Gorelik L, Forrestal F, Sandrock AW, E Goelz S. Assessment of JC virus DNA in blood and urine from natalizumab-treated patients. Ann Neurol. 2010 Sep;68(3):304-10.
242. Rinaldi L, Rinaldi F, Perini P, Calabrese M, Seppi D, Grossi P, Mattisi I, Barzon L, Mengoli C, Sanzari M, Palú G, Gallo P. No evidence of JC virus

reactivation in natalizumab treated multiple sclerosis patients: an 18 month follow-up study. J Neurol Neurosurg Psychiatry. 2010 Dec;81(12):1345-50.
243. Jilek S, Jaquiéry E, Hirsch HH, Lysandropoulos A, Canales M, Guignard L, Schluep M, Pantaleo G, Du Pasquier RA. Immune responses to JC virus in patients with multiple sclerosis treated with natalizumab: a cross-sectional and longitudinal study. Lancet Neurol. 2010 Mar;9(3):264-72.
244. Sadiq SA, Puccio LM, Brydon EW. JCV detection in multiple sclerosis patients treated with natalizumab. J Neurol. 2010 Jun;257(6):954-8.
245. Laroni A, Giacomazzi CG, Grimaldi L, Gallo P, Sormani MP, Bertolotto A, McDermott JL, Gandoglia I, Martini I, Vitello G, Rinaldi F, Barzon L, Militello V, Pizzorno M, Bandini F, Capello E, Palù G, Uccelli A, Mancardi GL, Varnier OE. Urinary JCV-DNA testing during natalizumab treatment may increase accuracy of PML risk stratification. J Neuroimmune Pharmacol. 2012 Sep;7(3):665-72. doi: 10.1007/s11481-012-9366-z.
246. Delbue S, Sotgiu G, Fumagalli D, Valli M, Borghi E, Mancuso R, Marchioni E, Maserati R, Ferrante P. A case of a progressive multifocal leukoencephalopathy patient with four different JC virus transcriptional control region rearrangements in cerebrospinal fluid, blood, serum, and urine. J Neurovirol. 2005 Feb;11(1):51-7.
247. Pagani E, Delbue S, Mancuso R, Borghi E, Tarantini L, Ferrante P. Molecular analysis of JC virus genotypes circulating among the Italian healthy population. J Neurovirol. 2003 Oct;9(5):559-66.
248. Agostini HT, Ryschkewitsch CF, Stoner GL. Genotype profile of human polyomavirus JC excreted in urine of immunocompetent individuals. J Clin Microbiol. 1996 Jan;34(1):159-64.
249. Ault GS, Stoner GL. Human polyomavirus JC promoter/enhancer rearrangement patterns from progressive multifocal leukoencephalopathy brain are unique derivatives of a single archetypal structure. J Gen Virol. 1993 Aug;74 (Pt 8):1499-507.
250. Delbue S, Elia F, Carloni C, Tavazzi E, Marchioni E, Carluccio S, Signorini L, Novati S, Maserati R, Ferrante P.JC virus load in cerebrospinal fluid and transcriptional control region rearrangements may predict the clinical course of progressive multifocal leukoencephalopathy. J Cell Physiol. 2012 Oct;227(10):3511-7. doi: 10.1002/jcp.24051.
251. Bozic C, Richman S, Plavina T, Natarajan A, Scanlon JV, Subramanyam M, Sandrock A, Bloomgren G. Anti-John Cunnigham virus antibody prevalence in multiple sclerosis patients: baseline results of STRATIFY-1. Ann Neurol. 2011 Nov;70(5):742-50. doi: 10.1002/ana.22606.
252. Bonig H, Wundes A, Chang KH, Lucas S, Papayannopoulou T. Increased numbers of circulating hematopoietic stem/progenitor cells are chronically maintained in patients treated with the CD49d blocking antibody natalizumab. Blood. 2008 Apr 1;111(7):3439-41.
253. Zohren F, Toutzaris D, Klärner V, Hartung HP, Kieseier B, Haas R. The monoclonal anti-VLA-4 antibody natalizumab mobilizes CD34+ hematopoietic progenitor cells in humans. Blood. 2008 Apr 1;111(7):3893-5.

254. Lindberg RL, Achtnichts L, Hoffmann F, Kuhle J, Kappos L. Natalizumab alters transcriptional expression profiles of blood cell subpopulations of multiple sclerosis patients. J Neuroimmunol. 2008 Feb;194(1-2):153-64.
255. Bennett JL. Natalizumab and progressive multifocal leukoencephalopathy: migrating towards safe adhesion molecule therapy in multiple sclerosis. Neurol Res. 2006 Apr;28(3):291-8.
256. Ransohoff RM. Natalizumab and PML. Nat Neurosci. 2005 Oct;8(10):1275.
257. Del Valle L, White MK, Khalili K. Potential mechanisms of the human polyomavirus JC in neural oncogenesis. J Neuropathol Exp Neurol. 2008 Aug;67(8):729-40.
258. Bossolasco S, Calori G, Moretti F, Boschini A, Bertelli D, Mena M, Gerevini S, Bestetti A, Pedale R, Sala S, Sala S, Lazzarin A, Cinque P. Prognostic significance of JC virus DNA levels in cerebrospinal fluid of patients with HIV-associated progressive multifocal leukoencephalopathy. Clin Infect Dis. 2005 Mar 1;40(5):738-44.
259. García de Viedma D, Alonso R, Miralles P, Berenguer J, Rodriguez-Créixems M, Bouza E. Dual qualitative-quantitative nested PCR for detection of JC virus in cerebrospinal fluid: high potential for evaluation and monitoring of progressive multifocal leukoencephalopathy in AIDS patients receiving highly active antiretroviral therapy. J Clin Microbiol. 1999 Mar;37(3):724-8.
260. Fox R. Advances in the management of PML: focus on natalizumab. Cleve Clin J Med. 2011 Nov;78 Suppl 2:S33-7.
261. Schwab N, Höhn KG, Schneider-Hohendorf T, Metz I, Stenner MP, Jilek S, Du Pasquier RA, Gold R, Meuth SG, Ransohoff RM, Brück W, Wiendl H. Immunological and clinical consequences of treating a patient with natalizumab. Mult Scler. 2012 Mar;18(3):335-44.

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Published abstract:

- Delbue S, Elia F, **Carloni C**, Colombo E, Gastaldi M, Franciotta D, Tavazzi E, Marchioni E, Bergamaschi R, Ferrante P. JCV and BKV urinary excretion increases during treatment with Natalizumab. Journal of Neurovirology, 2012, vol. 18, p. 30-31, ISSN: 1355-0284.
- Tremolada S, Delbue S, Elia F, **Carloni C**, Larocca S, Tavazzi E, Marchioni E, Bargiggia V, Bergamaschi R, Ferrante P. JCV and BKV urinary excretion increases during treatment with Natalizumab. Journal of Neurovirology, 2010, vol. 16, p. 88, ISSN: 1355-0284, doi: 10.3109/13550284.2010.522868.
- Elia F, Delbue S, Tremolada S, Larocca S, **Carloni C**, Tavazzi E, Marchioni E, Novati S, Maserati R, Ferrante P. Evaluation of biomarkers for the prognosis and diagnosis of different forms of leukoencephalopathies: a longitudinal study. Journal of Neurovirology, 2010, vol. 16, p. 30, ISSN: 1355-0284, doi: 10.3109/13550284.2010.522868.
- Delbue S, Tremolada S, Elia F, **Carloni C**, Bargiggia V, Tavazzi E, Bergamaschi R, Ferrante P. Study of Human Polyomaviruses reactivation in Multiple Sclerosis patients during Natalizumab therapy. Journal of Neurovirology, 2009, vol. 15, p. 23-24, ISSN: 1355-0284, doi: 10.1080/13550280903016074.

Abstract presented at conferences:

- Tremolada S, Delbue S, Elia F, **Carloni C**, Larocca S, Tavazzi E, Bargiggia V, Bergamaschi R, Ferrante P. JCV and BKV urinary excretion increases during the treatment with Natalizumab. 26th ECTRIMS; 13-16 Ottobre 2010, Gothenburg, Svezia.